SEPARATION AND CHARACTERIZATION OF OXYNTIC CELLS FROM FROG GASTRIC MUCOSA

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To my wife and children, for whom this work represents invaluable sacrifices.

To my parents, whose life and work will be always an ideal to pursue.

To Dick Durbin, from whom I not only learned science but what a scientist should be.

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ABSTRACT

In the present dissertation a method for obtaining viable, separated oxyntic cells is described. Cells are characterized from anatomical, physiological and biochemical studies. Surface epithelial cells and mucous neck cells are removed by osmotic shock, blotting and scraping. The remaining tissue is exposed to pronase (0.18%) on the luminal side. Cells are then released by shaking in a vortex mixer. Isolated cells are purified by a simple velocity sedimentation procedure. The final preparation contains 81.3 ± 4.5% (n=14) oxyntic or acid secreting cells, with a viability higher than 90%. Cells consume oxygen at a rate of 4.6 - 4.8 µl/mg d w hr. This rate is increased more than threefold by DNP and blocked by 70% by amytal. Histamine increases respiration by 7.4%, acetylcholine by 21.1% and db-cAMP by 40%. Pentagastrin has no effect. The QO_2 responses to acetylcholine and db-cAMP are inhibited by preincubation in SCN^- or Cl^--free solutions. Ultraestructural studies showed that intracellular structures are well preserved in isolated cells. Stimulation with db-cAMP induces a proliferation of surface membrane at the expense of intracellular tubulo-vesicular membranous components. In most cells this fusion is almost complete. These studies were confirmed by the use of ectoenzymes to monitor changes in surface area; oxygen consumption and surface phosphatase activity were highly correlated when cells were stimulated with histamine, acetylcholine and db-cAMP. Isolated cells were able to maintain normal concentrations of high energy phosphate compounds. Stimulation induced changes in these compounds that partially resembled those produced in intact mucosa. There was a decrease in ATP and PCr with increases in ADP, AMP and Creatine. This resulted in decreases in ATP/ADP, ATP/AMP and PCr/Cr ratios induced by

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stimulation. In K^+-free solutions, these effects were reversed and stimulation induced a net synthesis of ATP with an increase in ATP/ADP ratio. Oligomycin completely abolished the respiratory response to db-cAMP without effect on DNP induced respiration. These effects on respiration were accompanied by a reduction in PCr and ATP levels with a concomitant increase in ADP, AMP and creatine. Stimulation of oligomycin pretreated cells with db-cAMP induced a further decrease in PCr and ATP with increases in ADP, AMP and creatine. Intracellular pH, as measured by the DMO technique was 7.187, a value slightly above the extracellular pH (7.110). Stimulation with 5 mM db-cAMP brought about an increase in intracellular pH measured 20 minutes after addition and that was maintained at 60 minutes. This change was of about 0.14 pH units. The increase brought about by db-cAMP was abolished by pretreatment with SCN^- for one hour. The results presented indicate that oxyntic cells isolated by these methods are able to secrete hydrochloric acid. Furthermore they provide some information about cellular mechanisms of stimulation, production and regulation of the H^+ secretory process.

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Introduction

For more than a century investigators have been interested in the production of hydrochloric acid by the stomach of the vertebrate. Although a considerable body of information has been derived from these investigations, solutions to some important basic questions have not been obtained: 1) the role and mechanism of action of hormones in the stimulation of gastric secretion; 2) the interrelationship between secretagogues; 3) the coupling of energy metabolism to H^+ translocation; 4) cellular mechanisms for activating the secretory process; and 5) fine regulation at the molecular level. Elucidation of some of these operations would contribute to the understanding and potential treatment of some altered physiological states. Even if this were not the case, the gastric mucosa's exceptional capacity for transporting ions serves as a model for studying transepithelial, polarized transport. Furthermore, knowledge elicited from these studies may help in the understanding of the mechanisms and regulation of a number of biological processes in addition to solute translocation; such as stimulus-secretion coupling, membrane biosynthesis and turnover.

Most of the previous investigations have been carried out in isolated mucosal preparations or the in vivo mammalian stomach. However, when a particular process that occurs in a single cell type is studied, these preparations have disadvantages. For example, in vivo mammalian stomach is subject to the influence of a very complex interrelationship of vascular, neural and humoral factors. Thus, HCl secretion, the end product, is the result of innumerable balanced, ongoing processes. Furthermore, although no longer subjected to humoral and nervous influence from outside, the amphibian mucosal preparation in the Ussing chamber is a heterogeneous population of cells, including oxyntic cells, mucous neck cells, surface epithelial cells, and argentaffin cells. A varying number of connective tissue and smooth muscle cells are present depending on the type of preparation. Although these cells perform quite different functions, interactions between the different types of cells do occur and are important in the overall functioning of the gastric epithelium as an organ.

The diversity of cell types evidently creates uncertainty regarding conclusions drawn from biochemical assays of the intact mucosa. For example Harris et al. (1) found an increase in cAMP levels in the intact mucosa upon stimulation with theophylline. The addition of this compound is likely to raise cAMP levels in all cell types present since it inhibits the cyclic nucleotide phosphodiesterase (2,9). On the other hand, they were unable to record an increase in cAMP levels in response to histamine. Thus, although histamine may have induced an increase in cAMP levels in the oxyntic cell, the effect may have been masked by a decrease in cAMP level in some other cell type, such as the smooth muscle cell. Innumerable examples of this kind are found throughout the literature where conflicting results are produced because of the presence of different cell types. Consequently, it is reasonable to study physiological mechanisms concerning a given cell type in isolated cell preparations. It is of particular importance that the gastric physiologist have on hand a preparation of isolated viable cells in order to study some mechanisms of production and regulation of hydrochloric acid at the cellular or subcellular level.

Cells have been separated from a number of tissues by diverse techniques. In some of these tissues, isolation of cells has been followed by an exhaustive investigation of the functional characteristics of the cell preparation as compared with those of the intact tissue. Sometimes these studies have provided valuable information regarding physiological processes operative in that particular cell type. Thus, isolated cells provide a tool for physiological and biochemical investigations at the cellular, subcellular and molecular level without the interference of other elements.

Isolated oxyntic or acid-secreting cells have been obtained in more or less pure preparations from both mammalian and amphibian stomachs. Efforts, however, to ascertain their properties have been limited. By using mechanical trituration in citrate solutions, Bre Miller (3) attempted to separate cells from rabbit stomachs, but she was unsuccessful because of cell clumping.

Several groups have used proteolytic enzymes to separate cells. By using collagenase digestion of the rabbit gastric mucosa and centrifugation in a discontinuous Ficoll gradient, Walder and Lunseth (4) obtained a preparation that contained 30% to 50% parietal cells. MacDogual and DeCosse (5) employed a similar method to produce a preparation that contained 95% parietal cells. These authors reported that the use of an electric field oriented isolated cells in a polarized fashion at an interphase. They observed a net Cl^- movement from the "serosal" to the "mucosal" side of the sheet of cells. These potentially interesting experiments, however, are subject to criticism. For instance, the authors did not state whether intercellular functions had been reestablished, which, nevertheless, is precluded because of the use of Ca^{++}-free solutions (6). On the other hand, these studies were done without the exogenous addition of a secretagogue, and it is known that the mammalian stomach transports very little amounts of Cl^- in the resting state.

The first attempt to characterize isolated parietal cells was that of Croft and Ingelfinger (7) who used enzymatic digestion with pronase and collagenase. They measured oxygen uptake by the isolated cells and observed that it was not stimulated by histamine although it was dependent on the percentage of parietal cells. These cells were able to maintain intracellular electrolyte concentrations similar to those found in intact tissue (8). An attempt was made to measure changes in intracellular pH: a value of 7.28 was obtained by using the DMO method, but the expected change was not observed after adding histamine.

By using pronase digestion of the mucosa, Blum et al. (10) were the first to obtain isolated oxyntic cells from amphibian stomachs. They reported producing a rather pure preparation (80%) with high viability ($>$ 90%). In this preparation oxygen uptake was relatively rapid and dependent on the percentage of oxyntic cells. However, no studies with histamine or another secretagogue were reported. Microelectrode studies of the potential difference, and measurement of intracellular electrolyte concentrations demonstrated that these cells were capable of maintaining electrochemical gradients across the plasma membrane.

The effects of enzymic hydrolysis in biomembranes are well documented (for a thorough review see ref. 11). They include changes in overall topography and viscoelastic properties, immunological characteristics, asymmetrical distribution of molecules, permeability barriers and effects on the catalytic behavior of membrane-bound proteins. From these observations it becomes obvious that enzyme-separated cells may not be able to perform certain functions. Thus, it is necessary to ascertain whether these cells are capable of carrying out certain physiological processes believed to be characteristic of that cell type in the intact tissue.

In this study, secretion of hydrochloric acid is of primary concern, in turn presenting some difficulties in the assessment of ion transport. Where the secreted species is a protein, as in isolated pancreas exocrine cells, measurement of the biological response is no more difficult than in the intact organ. Thus, secretion of enzymes and hormones has been demonstrated in a variety of isolated cell systems including exocrine pancreas (12-14), isolated pancreas islets, thyroid cells (15), adrenal cells (16), and many others. Sometimes the process involves the transport of a molecule that can be radioactively labeled or a nonmetabolizable analog that can be measured (17). At other times, the biological response may not involve the transport of a given species but a change in some metabolic process that can be quantitated easily.

None of these is the case for the secretion of H^+ by the isolated oxyntic cells. When an H^+ is pumped at the apical side, an OH^- or HCO_3^- is released at the serosal side of the cell (18). Therefore, the secreted H^+ is immediately neutralized in the isolated cell suspension. Thus, the study of the secretory properties of isolated oxyntic cells must rely on some indirect indices of activity that correlate well with acid secretion in the intact mucosa.

This study reports on the separation and certain biochemical, physiological and morphological properties of oxyntic cells and their relationship to acid secretion by such cells.

The choice of properties to be studied was made somewhat arbitrarily in order to cover a broad spectrum of characteristics. It was thought that the study of a single parameter would not lead to an unequivocal conclusion regarding the secretory abilities of these cells. On the other hand, it was hoped that these investigations would provide important information about various aspects of the intact secretory machinery.

The aspects to be studied were: oxygen consumption, ultrastructure, adenine nucleotides and intracellular pH. The following paragraphs supply the rationale for the study of the preceding elements.

Acid secretion is dependent on oxidative metabolism. Furthermore, in a number of studies a close correlation has been demonstrated between oxygen uptake and transport activity (19-26). Addition of secretagogues leads to an increase in H^+ secretion and oxygen uptake. In a recent report Hersey (27) showed that the dose response curves for oxygen consumption and acid secretion, in response to theophylline, are identically parallel. Thus, measurement of the increase in respiration as affected by secretagogues may be employed as an index of secretory activity.

Ultrastructural changes that take place in the oxyntic cell in the transition from the resting to the secreting state are well documented (28,29). The apical side of the cell is occupied by a system of smooth surface tubulo-vesicular membranes which seems to fuse with the apical plasma membrane upon stimulation of acid secretion and in this way greatly increases the surface area of the apical plasma membrane (30). Evidence has been presented that identifies the tubulo-vesicular membrane system and the plasma membrane on the basis of membrane measurements (31,32), carbohydrate staining (33,34), asymmetry of the glycoprotein coat (35), and enzyme histochemistry (36). In addition, there is sufficient proof of the dynamic continuity of the tubulo-vesicular membranes and the plasmalemma (30,37,38). Depending on the physiological state, this con-

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tinuity may involve the fusion and the retrieval of membranes. Thus, this structure may represent a functional system in dynamic equilibrium in which flow in and out depends on the secretory activity. Studying these transformations in isolated oxyntic cells may provide information on the capability of the isolated cell preparation to secrete HCl. In addition, these studies could influence our understanding of vectorial transport processes across epithelial membranes.

Durbin and collaborators (39,40) have reported that concentrations of certain nucleotides are modified by stimulation. Transition from the resting to the secreting state brings about a consistent increase in ADP level with a decrease in the ATP/ADP ratio. In addition, phosphocreatine (PCr) levels are increased, creatine (Cr) decreases and consequently the PCr/Cr ratio increases also. These changes are dependent on the functioning of the H^+ pump since they are reversed by the inhibition of acid secretion by Cl^--free solutions, K^+-free solutions, and ouabain. The study of nucleotides in isolated cells is important for a number of reasons: 1) concentrations of adenide nucleotides would indicate whether these cells are able to metabolize normally; 2) changes in nucleotide ratios upon stimulation are indices of secretory activity; 3) if the ratios are altered, as in the intact mucosa, these changes occur in the oxyntic cell; and 4) the nature of the modifications provides some insight into the mechanisms of energy-secretion coupling.

It has been established that in the steady state the secretion of gastric acid into the lumen is accompanied by an stoichiometric release of alkaline into the blood or serosal side (18). Furthermore, Rehm and collaborators (41,42) have shown that the release of alkaline takes place by means of an electrically neutral exchange, probably of HCO_3^- by Cl^-.

Since H^+ pumping is an active process and alkaline release through the exchange mechanism is supposed to be passive, accumulation of base at the onset of acid secretion may be expected. This may then set up an electrochemical gradient that would drive the exchange mechanism. The initial accumulation of base (OH^- or HCO_3^-) would tend to shift the pH of the cytoplasmic compartment of the cell to the basic side. The magnitude of this shift will depend on the acid output, the volume, the buffering capacity of the cytoplasmic compartment, and the maximum rate of turnover of the exchange diffusion carrier. Change in cytoplasmic pH may be large enough to be measured by conventional methods such as the examination of the distribution of weak acids. It is assumed that the modifications in intracellular pH would be initiated by the active extrusion of protons from the oxyntic cells. Thus, intracellular pH would serve as an index of secretory activity.

In essence the work described in this thesis deals with the following: 1) the separation and purification of viable oxyntic cells from bullfrog gastric mucosa, and 2) the broad characterization of the properties of these cells from anatomical, biochemical and physiological studies.

II. METHODS

A) Solutions

Buffer solutions (TES-Nutrient) used in this study contained in mM: 70 NaCl, 4.0 KCl, 0.8 $MgCL_2$, 1.0 $NaHPO_4$,40.0 TES, 11 glucose.

TES-HCO_3^- Nutrient was prepared by replacing 18 mM TES (from a total of 40 mM TES) by 18 mM $NaHCO_3$; all other salts remained as in TES-Nutrient. Cl^--free solutions were prepared by substituting Na-isethionate or Na-glucuronate for NaCl. The chloride salts of K^+, Mg^{++} and Ca^{++} were replaced by the corresponding sulfate salts. In K^+-free solutions, KCl and penicillin were omitted.

"Enzyme solution" contained in addition to the above mentioned components, 5% fetal calf serum (Gibco) and 0.175%Pronase (Merch, 70000 u/mg). All solutions contained 1% bovine serum albumin (Miles Labs.) 100 u/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B except when stated.

B) Cell Isolation

The stratification of cellular types in the gastric mucosa of the bullfrog (Figure 1) makes feasible a fractionated isolation of different kinds of cells. However since the prime interest was in oxyntic cells, the method to be described applies only to this cell type.

A series of steps is employed to isolate oxyntic cells: 1) the surface epithelial cells and the mucous neck cells are removed; 2) the cells are isolated from the residual tissue; and, 3) the suspended cells are purified further so that they will yield an enriched fraction of isolated oxyntic cells.

The stomachs of large bullfrogs (Rana catesbeiana; 5-6 inches) of either

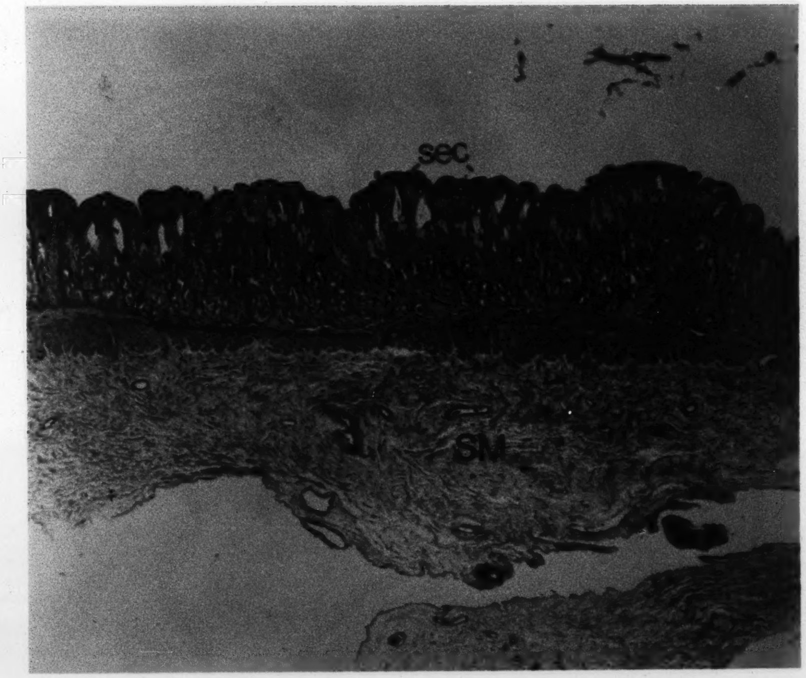

Figure 1: Section of gastric mucosa stripped from outer muscular coat. Surface is lined by surface epithelial cells (SEC). Two or three gastric glands (GG) empty into pits (P). Glands are formed by mucous neck cells (MNC) and oxyntic cells (OC). MM, muscularis mucosa. SM, submucosa. Stained with P.A.S. and P.T.A.H.

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sex are used for the isolation of cells. After pithing,the stomachs are removed quickly. They are opened through the lesser curvature, and the outer muscle coat is stripped from the mucosal layer. The latter, which will be referred to as mucosa, includes secretory glands, muscularis mucosa and a variable amount of connective tissue (Fig. 1).This is tied like a diaphragm at the end of a Lucite tube, with the serosal side facing the inside and the mucosal side facing the outside. Nutrient solution is added to the serosal side (inside of the tube) and oxygenated. No treatment whatsoever is done on the serosal side except for the periodic renewal of solutions.

Then the tube with the tied mucosa is immersed in a beaker containing different solutions according to the step to be performed, as shown in Figure 2A. The mucosa is washed twice in oxygenated frog Ringer's solution (nutrient) and then the assembly is put in a 25° water bath.

Essentially, Forte's et al. (43) procedure is used to remove the surface. epithelium and mucous neck cells. The luminal surface is exposed to a hypertonic solution (0.5m NaCl) for 10 min, both solutions are then drained and a piston is introduced in the tube in order to keep the mucosa in place, as is shown in Figure 2B. Next the luminal surface is blotted and scraped lightly with a scalpel. Then the mucosa is put back in the beaker with isosmotic nutrient solutions bathing both sides, and 15 min later blotting and scraping are repeated. This procedure selectively removes nearly all the surface epithelium and mucous neck cells, as has been demonstrated by Forte et al.

At this time, the luminal side is exposed to a nutrient solution containing 0.18% Pronase (Merck) and 5% fetal calf serum (GIBCO). The temperature is raised to 32°C and shaking in the water bath is begun.

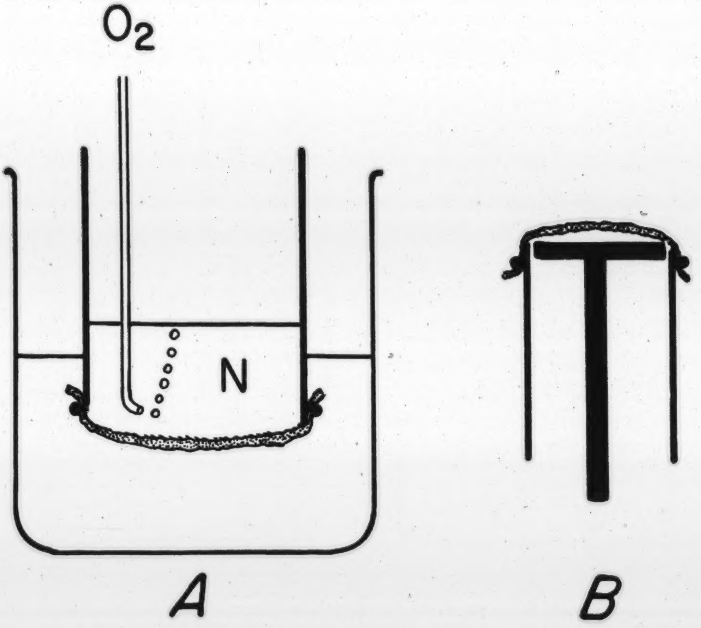

Figure 2: A. Schematic diagram of experimental set-up for the isolation of oxyntic cells. The mucosa is tied as a diaphragm at the end of a lucite tube. Nutrient solution is instilled on the serosal side (N) and oxigenated. Diverse solutions are instilled on the lumen side according to the step to be performed. B. Arrangement for blotting and scraping. Mucosa is supported by piston introduced on serosal side. See text for details.

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After 20 min the enzyme solution is removed and discarded, and the luminal surface is blotted lightly with filter paper. This solution contains debris, some cells and a visible amount of mucous. A fresh enzyme solution is instilled in the luminal side. After 60 min of exposure to the new enzyme, the assembly is shaken in a vortex-mixer intermittently for 2 min. This procedure brings about a massive detachment of cells from the mucosa. The suspension of cells in the enzyme solution is then spun down at 60 g for 5 min in a refrigerated centrifuge at 0°C (Sorvall RC2-B). The supernatant (enzyme solution) is drained, the pellet is resuspended in 10 ml of nutrient solution containing 10% fetal calf serum and then is stored at 4° C. New enzyme is instilled on the luminal side and the process of shaking in the vortex mixer is repeated once or twice at 30 min intervals until most of the cells become detached from the mucosa.

Usually four to six mucosae are processed at the same time. All the pooled cells are washed once again with nutrient + 10% fetal calf serum and resuspended in 5 ml nutrient + 1% albumin. This suspension is then layered on 7 ml of ice-cold nutrient + 10% fetal calf serum + 2% albumin and for 3 min it is spun down at low speed in a desk-top centrifuge equipped with a swinging bucket rotor (International Clinical Centrifuge Model Cl; speed "1"). By using this procedure,three fractions can be recovered: 1) a cloudy band containing debris and broken cells at the interface where the cells were layered; 2) a pellet corresponding to oxyntic cells at the bottom; and 3) a diffuse band spread through the denser medium. The last group consists of primarily non-oxyntic cells. If the yield needs to be improved, the suspended cells can be spun down and the entire procedure can be repeated.

C) Cell Identification

Identification of the cells is done by means of two techniques: phase-contrast microscopy and histochemistry.

Under the phase-contrast microscope two kinds of cells can be distinguished: large cells (20-25 μ) with dense granular cytoplasm and smaller cells (10-15 μ) with translucent cytoplasm. A few clumps of three to four cells are usually seen in the suspension.

Mitochondria are stained for further identification. Oxyntic cells are rich in mitochondria so it is possible to make a differential count for positive (oxyntic) cells. Succinic dehydrogenase is stained by a modification of the methods of Nachlas et al. (44). One ml of cell suspension is incubated at 37^o C for one to two hr with 1 ml of reaction medium containing 70 mM Na succinate, 2 mg/ml P-nitrotetrazolium blue and 2 mM azide.

Positive cells are densely packed with granules as shown in Figure 3. The positive cells correspond to the larger cells seen under phase-contrast, whereas the cells that do not stain correspond to the smaller cells.

Viability was determined by the trypan blue exclusion test.

D) Oxygen Consumption

Oxygen consumption (QO_2) of the cell suspension was measured in a glass chamber with magnetic stirring (Yellow Spring Instr. Co.) using Clark-type electrodes (Radiometer) connected to an amplifier and a recorder (Varian). The chamber was maintained at 25^o C with a circulating bath (Neslab). This set-up allowed for the simultaneous measurement of oxygen consumption in four different aliquots. Usually QO_2 was measured simultaneously in two chambers and then the electrodes were switched to the other two chambers. This procedure allowed time for equilibration with

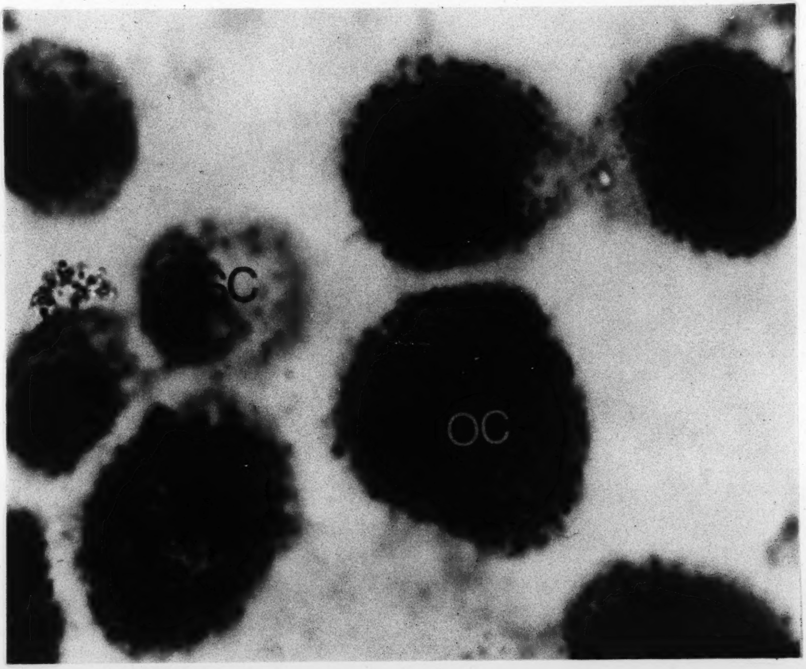

Figure 3: Identification of oxyntic cells by the nitrotetrazolium blue method. Oxyntic cells (OC) appear packed with dark granules. Non-oxyntic cells (SC), smaller in size contain very few granules. Nuclei stained with methyl green.

the gas phase in the aliquots not being measured.

Cells were suspended either in TES-nutrient or in HCO_3^--TES nutrient containing 1% Albumin at a concentration of about 1 mg dry weight/ml. From 3.5 to 4 ml were used in each chamber for the measurement of oxygen consumption. Suspensions were allowed to equilibrate for 20-30 min with the appropriate gas (air or 95% air-5% CO_2) before the measurements were started. In order to study the effects of different agents, QO_2 was measured two or three times before additions were made.

Oxygen consumption was calculated from the slope of the drop in PO_2 in the solution when the system was closed. PO_2 was never allowed to drop below 50% that of air. The results are expressed in µl/mg dry weight. In order to derive the dry weight of cells, samples of two or three ml of the cell suspension and the suspension medium were dried in an oven at 95°C in preweighed vials. The dry weight was obtained by subtracting the weight of the suspension medium from that of the cell suspension.

E) High Energy Phosphate Compounds

1) Preparation of Samples

a) Intact mucosa: The stomach of a pithed bullfrog was removed and the mucosa was separated from the muscular coat by means of blunt dissection. The mucosa was split into two halves and the paired segments were mounted in two separate sets of Lucite chambers using parafilm gaskets as described by Durbin (45). The serosal side (nutrient) was bathed with a solution that contained, im mM; 89.4 NaCl, 18 $NaHCO_3$, 4 KCl, 1.8 $CaCl_2$, and 0.8 $MgSO_4$ 11 glucose. The luminal (secretory) side was bathed with 120 mM NaCl. The H^+ secretory rate was monitored continuously by the pH-stat method (46) with an automatic buret and pH meter (Radiometer ABU 11 and TT11c).

The procedure of Kasbekar (47) was used to obtain resting mucosa. After mounting, solutions were renewed hourly until the spontaneous rate of acid secretion in both paired halves had declined below 0.5 µEq H^+/cm^2 per hr. At this point one-half of the pair was stimulated with 5 mM db-cAMP. The other half was kept as a resting control. One hr later, both halves were rapidly frozen (5 sec) in liquid Freon-12 (cooled with liquid nitrogen) and were maintained in this condition until extraction. The frozen samples were weighed and then ground to powder in a mortar chilled in liquid nitrogen. The frozen powder was placed on top of 0.3 ml of 3M $HClO_4$ in a conical test tube kept in dry-ice. The tubes were then transferred to an ethanol bath at -10°C. The samples were stirred with glass rods until the thawed percloric acid had penetrated the powder (three to five min). The tubes were then transferred to ice and 1.25 ml of 1 mM EDTA were added while the solution was being stirred. After five min the samples were centrifuged at 7700 g x 3 min, and 1 ml of supernatant was neutralized with 0.33 ml of 2M $KHCO_3$. After CO_2 had evolved completely, the samples were frozen in order to bring about the withdrawal of the dissolved CO_2, and then they were thawed and the supernatants were decanted from the $KClO_4$ precipitate and stored in dry ice.

b) Isolated cells: Isolated oxyntic cells were suspended in TES-Nutrient + 1% Albumin at a final concentration of one to two mg dw/ml. Aliquots of 3.5 ml were incubated in a glass chamber along with stirring open to the atmosphere (see QO_2 methods). After 10-15 min of incubation, stimulants (Acetylcholine, db-cAMP, and Histamine) were added to different aliquots, while one was kept as a control.

After one hr of stimulation, 3 ml of the cell suspension were poured into a conical test tube containing 0.15 ml 70% percloric acid (final concentration 3.3%). Then 0.5 ml 7mM EDTA were added and mixed and after five min they were centrifuged at 7700 g x 3 min. Three ml of supernatant were then neutralized with 0.9 ml of $KHCO_3$. Subsequent steps were the same as for intact mucosa.

Initial experiments demonstrated that ADP preferentially leaked out of the cells into the suspending medium. Thus, the following procedure was adopted. Three ml of the cell suspension were poured into ice-cold tubes containing 2 ml of the suspension medium. In this way the cells were rapidly chilled. The tubes were centrifuged at 0°C at 3000 g x one min, and the supernatant was drained and saved. One ml of 3.5% percloric acid (ice-cold) was added to the cell pellet and was shaken in a vortex mixer until the pellet had completely dissolved. Then 0.3 ml of 4 mM EDTA were added and shaken, and after two min the tubes were centrifuged at 7700 g x 3 min. One ml of supernatant was neutralized with 0.3 ml of 2 M $KHCO_3$. Subsequent steps were the same as for intact mucosa. The supernatant was processed in the same manner as for the cell suspension (see above) in order to measure nucleotide leakage from the cells.

2) Tissue Analyses

Percloric-acid extracts were used to measure ATP, ADP, AMP, phosphocreatine (PCr), and creatine (Cr). All analyses were performed fluorometrically, measuring the appearance of TPNH or the disappearance of DPNH, according to the methods described by Lowry *et al.*, (48,49).

a) ATP and PC

ATP and PC were measured sequentially in the same sample. The principle is as follows.

$$\mathrm{ATP + Glucose} \xleftrightarrow{\text{Hexokinase}} \mathrm{ADP + Glucose\text{-}6\text{-}P} \qquad (1)$$

$$\mathrm{Glucose\ 6\text{-}P + TPN^{+}} \xleftrightarrow[\text{dehydrogenase}]{\text{Glucose-6-P}} \mathrm{6\text{-}P\text{-}Gluconolactone} + \underline{\mathrm{TPNH}} + \mathrm{H^{+}} \qquad (2)$$

$$\mathrm{PCr + ADP} \xleftrightarrow{\text{Creatine Kinase}} \mathrm{Creatine} + \underline{\mathrm{ATP}} \qquad (3)$$

ATP was measured quantitatively by the appearance of TPNH and then comparing to a known ATP standard. When the reaction was completed (10 min), Creatine Kinase and ADP were added (Reaction 3) and the ATP that had been produced entered Reactions 1 and 2. The PCr was thus measured as a further increase in TPNH.

b) ADP-AMP

ADP and AMP were measured sequentially in the same sample according to the following principle.

$$\mathrm{ADP + P\text{-}Pyruvate} \xleftrightarrow[\text{Kinase}]{\text{Pyruvate}} \mathrm{ATP + Pyruvate} \qquad (4)$$

$$\mathrm{Pyruvate} + \underline{\mathrm{DPNH}} + \mathrm{H^{+}} \xleftrightarrow[\text{Dehydrogenase}]{\text{Lactic}} \mathrm{Lactate + DPN^{+}} \qquad (5)$$

$$\mathrm{AMP + ATP} \xleftrightarrow{\text{Myokinase}} \underline{\mathrm{2\ ADP}} \qquad (6)$$

ADP was measured quantitatively as a disappearance of DPNH (Reactions 4 and 5). After Reaction 5 had come to completion (10 min) and readings had been taken for ADP, myokinase was added, and the AMP present in the sample was converted to ADP (Reaction 6). The ADP which had been produced entered into Reactions 4, and 5, and the AMP was measured as the further disappearance of DPNH. Notice that two ADP's were produced from AMP, which serves as a magnification factor.

c) Creatine

Creatine was measured enzymatically according to the following

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principle:

$$\text{Creatine} + \text{ATP} \xleftrightarrow[\text{Kinase}]{\text{Creatine}} \text{PCr} + \text{ADP} \qquad (7)$$

$$\text{ADP} + \text{P-Pyruvate} \xleftrightarrow[\text{Kinase}]{\text{Pyruvate}} \text{ATP} + \text{Pyruvate} \qquad (8)$$

$$\text{Pyruvate} + \underline{\text{DPNH}} + \text{H}^+ \xleftrightarrow[\text{dehydrogenase}]{\text{lactic}} \text{lactate} + \text{DPN}^+ \qquad (9)$$

Creatine is measured quantitatively as a disappearance of DPNH (Reaction 9). In this method, Reactions 8 and 9 are allowed to proceed first, until all the ADP initially present in the sample has been exhausted (5-10 min). Then the creatine kinase is added, and the creatine is measured. In this manner the values for creatine do not have to be corrected for preexisting amounts of ADP in the sample.

Metabolite concentrations are expressed as nanoMoles/mg wet tissue for intact mucosa or nanoMoles/mg dry weight for isolated cells. For the purposes of comparison, the dry weight of cells can be multiplied by three (50) to obtain the wet weight of cells.

F) Light Microscopy

In order to monitor the different steps in the isolation procedure, intact mucosae in different stages were processed by conventional light microscopy procedures. Pieces of gastric mucosa were fixed in 10% formaldehyde and then in Zenker's fluid and embedded in parafin. Sections were stained with the periodic acid-Schiff reaction (PAS) and phosphotungstic acid hematoxylin (PTAH).

G) Electron Microscopy

In order to examine the effects of stimulation with db-cAMP on the morphology of oxyntic cells, experiments were performed on intact mucosa and isolated cells in both resting and stimulated conditions. These studies were performed with the kind help of the Laboratory of Clinical

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Pathology.

a) Intact Mucosa: Paired halves of the same mucosa were mounted in Ussing chambers as was described above (omitting, however, parafilm gaskets). After the spontaneous secretory rate had dropped to a resting value (0.5 uEq H^+/cm^2 hr), one-half was stimulated by adding db-cAMP to a final concentration of 5 mM. After one hr nutrient and secretory solutions in both halves were replaced by an ice-cold nutrient solution containing 3% glutaraldehyde, and oxygenated in the chamber for one min. Immediately afterward, the solution was drained and the exposed area of the mucosa was minced with a razor blade in a dish containing 3% glutaraldehyde solution. Then the minced tissue was suspended in 10 ml 3% glutaraldehyde in TES-Nutrient solution, and was fixed for three hr at 4°C. The tissue was washed in 0.1 M phosphate buffer and postfixed in OsO_4 for one hr; after which it was dehydrated in a series of alcohols, and embedded in araldite.

b) Isolated Cells. Isolated oxyntic cells were suspended in TES-Nutrient + 10% fetal calf serum; the cell suspension was incubated in a glass chamber and stirred. After an equilibration period (15-30 min), db-cAMP was added to one chamber while another one was kept as a control. Sixty min afterward, both batches of cells were fixed by the addition of an equivalent volume of 3% Glutaraldehyde in TES-Nutrient + 10% fetal calf serum. After undergoing fixation for three hr at 4°C; the cells were spun in plastic tubes in a microcentrifuge Eppendorf. The tips of the tubes were cut, and the pellets were withdrawn. The cell pellets were postfixed in 1% OsO_4 for one hr., dehydrated in a series of alcohols, and embedded in araldite.

Thin sections from both intact mucosae and cell pellets were cut with glass knives and examined in a SIEMENS ELMISKOP 1A electron microscope operated at 80 KV.

H) p-Nitrophenyl Phosphatase (pNPPase)

The observation of the surface activity of this enzyme was used to follow changes in membrane areas upon stimulation (51).

Isolated oxyntic cells were suspended in TES-Nutrient + 1% Albumin at an approximate concentration of 2.2 mg dry weight/ml. Four aliquots of five ml each were placed in the incubation chamber (cf. QO_2 Methods). After 30 min of incubation, pNitrophenyl-phosphate (SIGMA) was added to all four chambers at a final concentration of 0.5mM. After 10 minutes, Acetycholine (10^{-3}M), Histamine (10^{-4}M) and db-cAMP ($5x10^{-3}$M) were added to three different chambers, and one was kept as a control. Two minutes afterward, 1.2 ml samples were taken. After secretagogues had been present for 40 min, QO_2 was measured in the four chambers as was described above. Then 60 min after the addition of secretagogues, 1.2 ml samples were taken.

For the measurement of pNPPase activity, the product P-Nitrophenol was measured in each sample by means of the following method. Each sample was immediately spun at 7700 g x 1 min. One ml supernatant was mixed with one ml 0.05 NaOH and the optical density was read in a Coleman spectrophotometer at 405 manometers. In order to calculate the enzyme activity, the reading at 0 min was subtracted from that at 60 min. The activity is expressed in nMoles of p-nitrophenol per mg dry weight per hr.

I) Intracellular pH

In order to measure intracellular pH, a modification of the method of Poole et al (52) was used. This technique is based upon the equilibration of the weak acid 5,5-Dimethyl-2, 4-oxazolidinedione-^{14}C (DMO-^{14}C) across the membrane of the cell. If one assumes that only the undissociated form is permeable, then the intracellular pH can be calculated by measuring extracellular pH, DMO-^{14}C in the cell pellet, the supernatant, and the cellular volume (53).

In the method described by Poole et al. (52) two parallel pellets are analyzed; one for DMO-^{14}C and one for Inulin-^{14}C, each having a different extraction procedure.

In preliminary experiments it was found that this extraction procedure in two different pellets introduced a big source of error, up to 0.1 pH units, that limited the sensitivity of the method. Thus, the procedure was modified so as to allow for the simultaneous determination of DMO-^{14}C and Inulin-^{14}C in a single pellet.

Isolated oxyntic cells were suspended in TES-HCO_3^- nutrient + 1% albumin set approximately at a concentration of 2 mg dry weight/ml. DMO-^{14}C and Inulin-^{14}C were added to the cell suspension contained in a Falcon culture flask at final concentrations of 0.005 mCi and 0.1 mCi/ml of cell suspension respectively.

The suspension was incubated in a shaking bath for five min and then transferred to glass chambers with magnetic stirring (Yellow Springs Instruments) at 25°C. Air + 5% CO_2 was blown over the surface.

In experiments where the effect of stimulation on intracellular pH was studied, 7 ml of cell suspension was contained in each of two chambers. After an equilibration period of an hour, the pH of the cell suspensions was measured with a glass-calomel combination electrode and a pH-meter equipped with an expanded scale (Radiometer, PHM27). Then a 2 ml sample was placed in preweighed glass tubes; this was termed 0 min sample. Immediately after, 5 mM Dibutyryl-cAMP (db-cAMP) was added to one of the chambers. Samples were taken from control and experimental chambers at 20 and 60 min. Extracellular pH was measured prior to withdrawal of the samples.

Determination of DMO-^{14}C and Inulin-^{14}C

In order to determine the quantities of DMO-^{14}C and Inulin-^{14}C, each 2 ml sample was processed in the following way. Immediately after taking the sample, the tubes were spun at 3000 g for one min at 0°C in a Sorvall centrifuge (RC-2B) equipped with an angle rotor. The supernatant was removed, the inside walls of the tube were wiped dry, and the tube with the pellet was weighed. A 0.1 ml sample of the supernatant was then transferred to a glass test tube. The tubes containing the cell pellet and the supernatant sample were then dried in an oven at 95°C for 24 hr. The dried cell pellet was then weighed in order to determine the total water content of the cell sample. To both supernatant and cell sample, 1 ml of 0.1 N HCl was added and they were incubated for 36 hr at room temperature. After this period, the samples were spun at 7700 g x 3 min in order to remove the digested tissue. A 0.1 ml sample was taken from both cell and supernatant samples and was added to a scintillation vial containing 10 ml of a toluene-base scintillation fluid. In this sample total radioactivity contributed both by Inulin-^{14}C and DMO-^{14}C was measured. However, the counts due to Inulin were 20 times those contributed by DMO-^{14}C. In order to determine only DMO-^{14}C, alone, a procedure similar to that of Poole et al. was used except that two extractions were performed.

Five ml of 50% ethyl acetate-50% toluene (v/v) to the remaining 0.9 ml in the sample 50% toluene (v/v) were added. The tubes were shaken for 30 sec in a vortex mixer and then centrifuged at 3000 g x 3 min. Of the upper layer (ethyl acetate-toluene), four ml were transferred to a counting vial containing 10 ml of a toluene-base scintillation counting fluid. Another four ml of the ethyl acetate-toluene mixture were then added and shaking and centrifugation were repeated. Four ml from the upper layer was transferred to a counting vial as before. These samples were termed D_1 and D_2 and total DMO counts were calculated from D_1 and D_2 after correction for background.

The extraction of DMO by this method is essentially complete. Because inulin is not extracted into the organic phase, the inulin counts can be calculated by subtracting the DMO counts from the total counts.

Calculation of Intracellular pH

The equation described by Wadell and Butler (53) as modified by Poole et al. (52) was used to calculate intracellular pH. Direct counts are used in this equation.

$$\mathrm{pHi} = \mathrm{pK} + \log \left\{ \left[\frac{D_c}{D_s} \left(\frac{I_s}{I_s - I_c} \right) - \frac{I_c}{I_s - I_c} \right] \left[10^{\mathrm{pHe} - \mathrm{pK}} + 1 \right] - 1 \right\} \quad (10)$$

Where

pHi=intracellular pH

pHe=extracellular pH

D_c=total DMO counts in the cellular sample (calculated from D_1 and D_2), per mg water in cell pellet.

D_s=total DMO counts (from D_1 and D_2) in the supernatant sample per mg water.

I_c=Inulin counts in cellular sample per mg water after correction for DMO counts.

I_s=Inulin counts in supernatant sample per mg water after correction for DMO counts.

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III. RESULTS

A. Cell Isolation

The use of the procedure originally described by Forte et al. (43) was confirmed to give a preparation essentially free of epithelial cells. A few mucous neck cells, however, still were attached to the mucosa, but stronger scraping was not attempted. Probably most of the mucous neck cells are removed by the light blotting performed after 20 min of exposure to the enzyme. When the new enzyme solution has been present for 60 min, about half of the oxyntic cells have already become detached from the glands, but they are still immersed in the collagen mesh of the tissue, as is shown in Figure 4. Most of these cells come off the tissue when they are shaken in the vortex mixer.

At the end of the digestive and shaking procedure, only a thin layer of connective tissue and smooth muscle cells is left (Figure 5).

Included in the overall isolation are other steps that result in purification of the cell suspension. Centrifugation of the suspension in the enzyme solution at 60 g removes finer debris and soluble and suspended mucus that remains in the supernatant.

Oxyntic cells are larger (10) and denser (4,5), than other cells in the tissue; especially epithelial cells. These two characteristics can be exploited to perform a simple sedimentation rate separation.

The velocity of a relatively large spherical particle that moves through a stable medium depends on the field applied and the resistance to movement in the fluid. Resistance balances the acceleration due to the applied field; thus, the particle quickly reaches a constant velocity. In the case of cells, a centrifugal or gravitational field can be used.

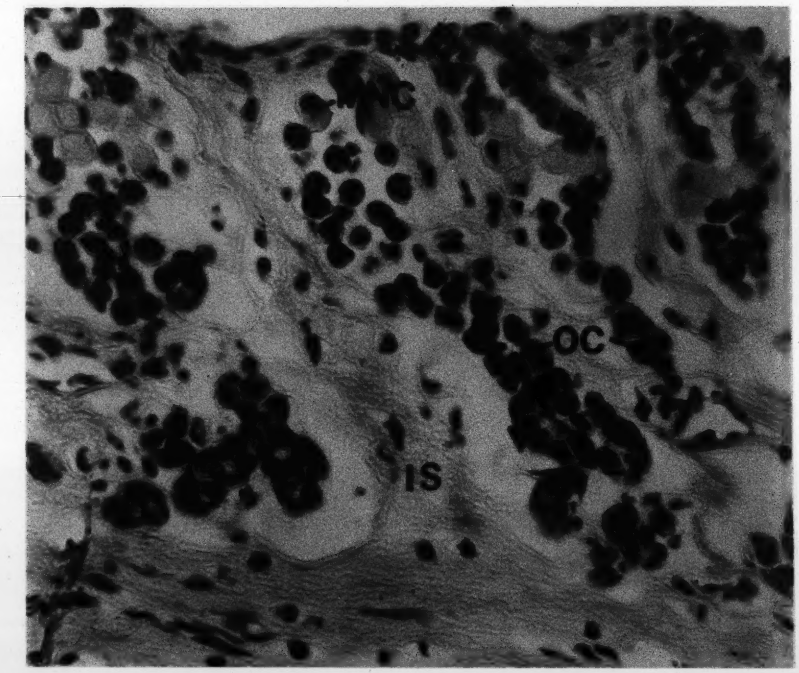

Figure 4: Section of gastric mucosa after treatment with pronase for 60 minutes, prior to agitation in vortex mixer. Surface epithelium has been scraped off (see text). MNC, mucous neck cells. Some oxyntic cells (OC) appear completely separated in the upper part of the gland. Interstitial space appears digested. Stained with P.A.S. and P.T.A.H.

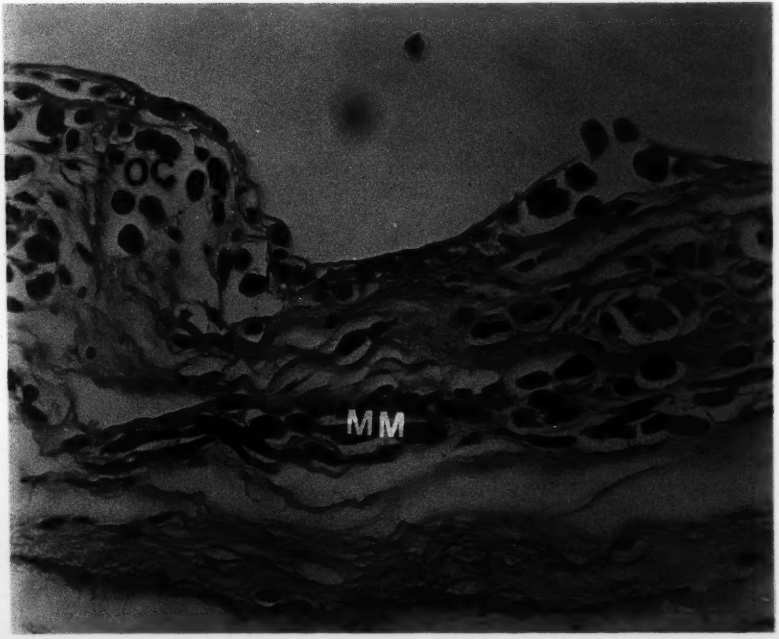

Figure 5: Section of gastric mucosa after separation of oxyntic cell has been performed. A few oxyntic cells (OC) remain immersed in the mucosal connective tissue. Some smooth muscle cells from muscularis mucosa (MM) become round, others retain their spindle shape. SM, submucosal connective tissue.

The velocity for the gravitational field is given by:

$$v=2(p-po)\ g\ r^2/9\eta \qquad (11)$$

Where v is equal to final velocity, p equals density of the cell, p_o equals density of the medium, g equals gravitational field, η equals viscosity of the medium, and r equals radius of the cell. The sedimentation velocity of a cell depends on its density and its diameter. Usually the differences in density are small in comparison with those in diameter. Moreover, there is poor resolution in the separation by size since all the cells sediment in a very short time.

The density factor becomes very important when the density of the cell approaches that of the medium. Also, more resolution is obtained by increasing the viscosity of the medium, and, in this way reducing the sedimentation velocity.

In the case of isolated cells from frog stomachs, the density factor can be exploited by increasing the density of the medium; in this case it was done by the addition of 2% albumin and 10% fetal calf serum. This procedure also increases the viscosity of the medium.

An increase in the density of the medium reduces the P-Po factor much more for the nonoxyntic cells than for the oxyntic cell fraction, in this way enhancing the difference in sedimentation velocity.

Equation 11 demonstrates the sedimentation velocity to be proportional to $r^2/4$. Thus, a 20 μ cell like the oxyntic cell, under the earth's gravitational field, would sediment four times faster than a 10 μ-diameter cell, even without taking into account the P-Po factor.

Sedimentation velocity separation under the earth's gravitational field has been used with good results in the present study. However, use of a centrifugal field has the advantage of being much faster. For this reason, centrifugation has been chosen as a routine procedure.

In this case velocity is not constant but is a function of the radial position. The equation becomes:

$$\frac{dx}{dt} = \frac{r^2(P-P_o)w^2x}{18\eta} \qquad (12)$$

Where w equals angular velocity and x equals radial position at time t.

For this study no attempts have been made to find a solution to this equation for the conditions employed; densities and centrifugal forces have been chosen from experimental trial.

As was mentioned before in the Methods, by using sedimentation rate separation the oxyntic cells sediment much quicker and can be recovered in the pellet, whereas smaller and less dense cells remain suspended by the time centrifugation is stopped (3 min). Although sometimes larger in size because of swelling, dead cells remain suspended at the interphase where the cells are layered in the denser medium, probably because of a decrease in cell density. $P-P_o$ would then approach zero and v would also approach zero. By using the preceding procedures a preparation can be obtained that contains more than 80% oxyntic or acid-secreting cells (81.3±4.5%; n=14) and with a viability always larger than 90%.

The total yield of cells from six frog stomachs is from 50 to 100 mgr dry weight of cells. Assuming a wet/dry ratio of three for isolated cells, 150 to 300 mg wet weight of cells are obtained. If a mean wet weight of 1500 mgr for six frog stomachs is assumed and taking 28% as the volume of oxyntic cells in the tissue (54), about 400 mgr of oxyntic cell is present in six frog stomachs. By using the upper value for cell yield, and multiplying it by 81.3 (the percentage of oxyntic cells in the isolated cell preparation), 245 mg of oxyntic cells are obtained representing about 60% of the oxyntic cells initially present in the tissue. The rest is lost probably during blotting, scraping, washing and purification procedures. Inclusion of proteins in the solutions is absolutely essential in order to preserve viability and prevent clumping.

B) Respiration

In control experiments oxygen consumption was found to be stable for periods of up to four hours. In order to gain some information about the general metabolical characteristics of the cell preparation, the effect of some metabolic inhibitors on oxygen uptake has been studied (Table I). Amytal inhibits 70% of the basal respiration. These results are similar to the ones obtained for intact mucosa (93) and may indicate that a substantial amount of respiration is linked to succinate oxidation. Dinitrophenol (DNP) increases respiration more than threefold, and the increase is larger than that observed in the intact mucosa (23,65), indicating that isolated cells are tightly coupled. The results using azide as an inhibitor were unexpected. This compound increases respiration in isolated oxyntic cells instead of acting as a blocker of electron transport. Thus, azide may act as an uncoupler in stomach as reported for other systems (85).

The results obtained in control conditions and after stimulation with different secretagogues are shown in Table II. If results obtained in control periods are pooled, basal oxygen consumption is 4.77 ± .11 ul/mg dry weight per hr (n=41). Basal respiration is fairly stable for long periods of time and is rather high compared to the oxygen utilized by pieces of intact mucosa. Presumably, we are dealing with a select

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TABLE I. Effect of metabolic inhibitors on oxygen uptake (QO_2) by isolated oxyntic cells. QO_2 measured before and after addition of inhibitor. n=number of experiments in each series. Cells pooled from 3-6 mucosae in each experiment.

		QO_2 (µl/mg dw. hr)			
	(n)	Control	Treated	$\frac{\text{Treated}}{\text{Control}}$	"p"
AMYTAL 2mM	(8)	4.37 ±.21	1.32 ±.17	0.30 ±.03	$<$ 0.001
AZIDE 2mM	(5)	4.64 ±.39	5.91 ±.6	1.27 ±.05	$<$ 0.01
DNP 0.1mM	(3)	4.90 ±.15	16.2 ±1.4	3.3 ±.2	$<$ 0.001

population of metabolically active cells.

Different secretagogues give rather different responses in respiration. Histamine increases respiration 7.4%, which is a statistically significant change. It should be noted that although the response is small (+ .35 $\pm$ 0.05 μl), it is distinctly different from the behavior of the controls (-0.05 $\pm$ 0.04 μl). Acetylcholine seems to be a more potent stimulator of respiration (21.1% increase) ($n=9$; $p<0.001$). In a short series of experiments, the effects of histamine and acetylcholine appear to be additive (28.8%) ($n=5$). At maximal concentrations Pentagastrin (10^{-6} M) does not stimulate respiration in this preparation. Dibutyryl-cAMP (db-cAMP) (5mM) is the most potent stimulant of respiration; it increases QO_2 by about 40%.

Response to histamine is small. As has been reported in the literature, the response, however, to this secretagogue varies depending on the laboratory and the conditions (Ref. 25). Consequently, the question can be raised as to whether the receptor has been damaged by the proteolytic enzyme treatment. In similar concentrations Pronase can degrade membrane proteins in other systems (11). Several experiments have been devised in an attempt to find an answer to this question. Kono (55) has reported that isolated fat cells that had lost their responsiveness to insulin after separation, recover biological response to the hormone after incubation at 37°C for two hr. Similar observations have been reported for isolated thyroid cells (56). Presumably the receptor had been resynthetized during that time. In the present preparation, 12 hr. of incubation of isolated oxyntic cells in a medium containing amino acids and serum did not change the response to histamine. In other types of exper-

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TABLE II. Effect of secretagogues on oxygen uptake (QO_2) by isolated oxyntic cells. See text for details. n=number of experiments in each series. Cells pooled from 4-6 mucosae in each experiment.

		QO_2 (µl/mg dw hr)				
	n	Before Treatment	After Treatment		%	"p"
CONTROLS	(11)	4.84 ±.23	4.79 ±.21	-0.05 ±.04	-1.2 ±.5	n.s.
HISTAMINE	(10)	4.75 ±.23	5.11 ±.26	0.35 ±.05	7.4 ±.9	<0.001
ACETYLCHOLINE	(9)	4.88 ±.28	5.91 ±.33	1.01 ±.07	21.1 ±2.0	<0.001
HISTAMINE + Ach	(5)	4.58 ±.24	5.90 ±.4	1.33 ±.28	28.8 ±5.8	<0.01
PENTAGASTRIN	(6)	4.70 ±.19	4.64 ±.2	-0.02 ±.05	-1.3 ±.03	n.s.
db-cAMP	(16)	4.74 ±.17	6.64 ±.3	1.88 ±.17	39.4 ±2.8	<0.001

iment, cells were isolated in the presence of large doses of histamine (10^{-3}M) or burimamide (10^{-3}M), an H_2-receptor antagonist (57),in order to "protect" the receptor.

None of these treatments modifies the respiratory response to histamine.

Since these experiments did not answer the question about receptor damage or regeneration, another approach was adopted: isolated mucosae were incubated in Ussing-type chambers in order to obtain resting stomachs. At this point the serosal side was exposed to a solution containing .18% pronase for one hr., then Pronase was thoroughly washed out and the tissue was stimulated with 10^{-4}M histamine. Acid secretion, potential difference and resistance were monitored continuously. The result of one such experiment is shown in Figure 6.

Addition of pronase to the serosal side of the mucosa produces an unexpected increase in acid secretion accompanied by a decrease in resistance and a small rise in the potential difference. These changes, however, are transitory and after reaching a maximum value about 20 min after the addition of pronase, all parameters begin to reverse. Acid secretion drops down almost to control values. After an initial rise, however, resistance again starts to decline and keeps declining even after Pronase is washed out. At this point, changes in potential difference parallel those in resistance. In spite of very low resistance and short-circuit current addition of histamine brings about an increase in acid secretion.

Early changes in acid secretion, potential difference, and resistance induced by Pronase are difficult to explain. Nevertheless, they resemble the changes induced by the addition of secretagogues, especially histamine (58). Thus, pronase may induce a release of histamine from stores

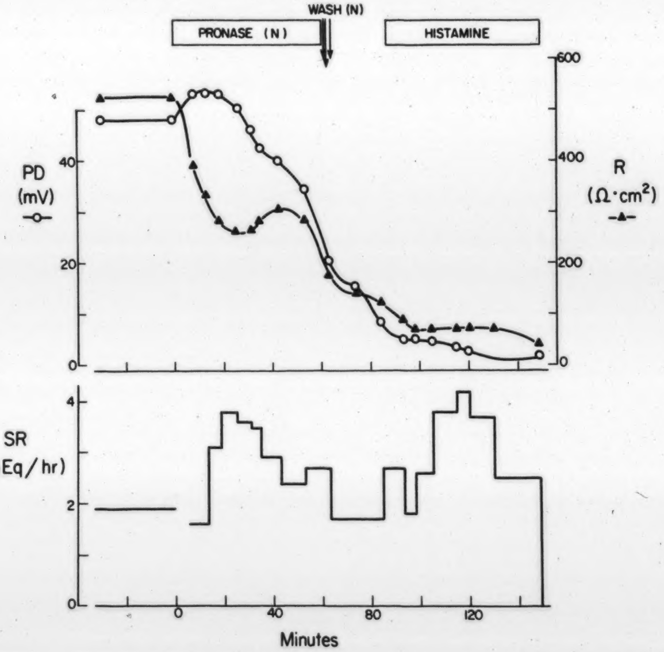

Figure 6: Effects of pronase on acid secretion (SR), potential difference (P.D.) and resistance (R) and on the subsequent response to histamine. Resting stomach in Ussing-chamber was exposed to 0.175% pronase for 60 minutes on the nutrient side. Pronase was then thoroughly washed out and histamine (10^{-4}M) was added to the nutrient side.

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outside the oxyntic cell as has been reported for pentagastrin, acetylcholine and barium (59,60), or it may induce the unmasking of receptors as it has been reported in other systems (61).

Subsequent changes in epithelial resistance and potential difference are explained by the opening of the intracellular junctions by the proteolytic enzymes. Even in this condition the response to histamine is large enough to be measured, in spite of probably a large back diffusion of H^+.

Although it was not conclusive, this experiment indicates that exposure of the serosal side to the enzyme for up to one hr to a large extent does not damage the histamine receptor.

None of these experiments, however, provides an answer about the status of the receptor after the isolation procedure.

An alternate explanation of high basal oxygen consumption and low response to histamine is that the unstimulated cells are not in the resting state. It is unlikely that high levels of histamine remain in the cell suspension after a number of washes. On the other hand, burimamide, an H_2-receptor antagonist (51,62), did not alter the level of basal respiration in three experiments. However, it is still a possibility that pronase irreversibly activates some receptors (61).

If secretagogue-induced respiration is directly associated with acid secretion by the isolated cells, it should also be blocked by known inhibitors of acid secretion. The most used inhibitors are SCN^- and Cl^--free solutions. The absence of Cl^- in the solutions presumably does not have any effect other than on the pump. Although the mechanism of action of SCN^- is not known, for a long time it has been considered to be a specific inhibitor of the H^+ pump in the stomach. This assumption is based on the observation that SCN$^-$ does not modify Cl$^-$ transport whereas the former inhibits acid secretion. In the present study both Cl$^-$-free solutions were studied using db-cAMP as a stimulant, whereas the effects of SCN$^-$ were studied using acetylcholine (10^{-3}M) and db-cAMP (5 x 10^{-3}M).

Table III presents the effects of preincubation in 20 mM SCN$^-$ for one hr on the respiratory response to acetylcholine (10^{-3}M) by isolated oxyntic cells. As usual (n=5), QO_2 is measured during the one hr control period, after which SCN$^-$ is added to one chamber. After one hr of incubation, acetylcholine (10^{-3}M) is added to both control and experimental chambers and respiration is measured.

Basal respiration does not change in the control chamber as was expected from the control studies (see above). The addition of SCN$^-$ does not modify basal oxygen consumption. Acetylcholine induces an increase in respiration of 23% above control values; although the increase is blocked partially by SCN$^-$. In this case acetylcholine-induced respiration is only 10% above basal QO_2.

Results obtained using db-cAMP as a stimulant are shown in Table IV. Respiration of the control is stable as in the acetylcholine experiments. Again, SCN$^-$ does not modify respiration in the isolated cell preparation. Db-cAMP stimulates QO_2 by 35.9% and again, stimulation of respiration is partially blocked by preincubation with SCN$^-$.

These results indicate that SCN$^-$, which inhibits acid secretion in the intact mucosa, is able to reduce the stimulation of respiration due to db-cAMP and acetylcholine. In turn the results suggest that a large portion of the stimulation of respiration is associated with acid secretion.

Since SCN$^-$ may not be a totally specific inhibitor of acid secretion at these concentrations, the effects of Cl$^-$-free* solutions were examined

*Cl$^-$ free solutions contained up to 1mm Cl$^-$ provenant from Albumin

TABLE III. Effect of SCN^- (20mM) pretreatment on respiratory response to Ach (1mM) in isolated oxyntic cells. QO_2 measured in both chambers in control conditions. Then SCN^- is added to one chamber for 1 hr and QO_2 measured at the end of the period. Then Ach (1mM) added to both chambers. (n=5; cells pooled from 4 mucosae in each experiment).

	QO_2 (µl/mg dw hr)				
	Control	SCN^- pretreat.	Ach	%	"p"
CONTROL	4.31 ±.10	4.49 ±.12	5.52 ±.23	23.0 ±2.9	0.001
SCN^-	4.55 .22	4.57 ±.29	5.08 ±.45	10.2 ±3.1	0.005
"p" (SCN^--Cont.)	n.s.	n.s.	< 0.01	<0.005	

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TABLE IV. Effect of SCN^- (20mM) pretreatment on the respiratory response of isolated oxyntic cells to db-cAMP (5mM). See Table III for experimental details. (n=3; cells pooled from 6 mucosae in each experiment).

	QO_2 (µl/mg dw hr)				
	Control	SCN pretreat	dbcAMP	3-2	%
CONTROL	4.23	4.35	5.91	1.56	35.9
SCN^-	4.13	4.54	5.35	0.81	17.4

by using db-cAMP as a stimulant. Isolated cells were divided into two aliquots and they were spun down. The pellets were resuspended either in TES-Cl nutrient or in TES-Isethionate nutrient containing 1% albumin. After ten minutes centrifugation was repeated and the pellets again were suspended in the above solutions and incubated further for two hours before measurements were made. Respiration was measured in control conditions and under stimulation with db-cAMP in both Cl^- and Cl^--free solutions.

Results obtained in six paired experiments are shown in Table V. Control respiration is significantly higher in Cl^--free solutions than in Cl^- solutions. The difference is not dependent on the nature of the substituting ion because identical results are obtained when glucoronate is substituted for Cl^-. These results do not agree with any of the earlier reports that respiration was not changed in Cl^--free solutions (25). Nevertheless, db-cAMP stimulates QO_2 to a larger extent when Cl^- is present in the solution, which agrees with Alonso's et al findings in intact mucosa (25). Both relative and absolute increases in respiration that are induced by db-cAMP are blocked by the absence of Cl^- in the solutions.

The results using Cl^--free solutions, as well as the observed effects on stimulation by SCN^- strongly suggest that at least a large fraction of secretagogue-induced respiration is associated directly with the stimulation of acid secretion in the isolated cell preparation.

The remaining fraction of secretagogue-induced respiration is most likely not associated with residual stimulation. The fact that the degree of inhibition in the three situations is about the same indicates that a process other than acid secretion is responsible for the increase in QO_2 elicited by secretagogues. A likely possibility is the stimulation of metabolic processes by cAMP. Exogenous addition of substrates has been shown to stimulate QO_2 by the gastric mucosa. In addition, the stimulation of glycolysis and lypolysis by cAMP is known to occur in the gastric mucosa (63-65) and other tissues (66). Thus, secretagogues in addition to stimulate the H^+ pump may induce an increase in substrate metabolism by making them available to oxydation. In other words secretagogues may promote "substrate mobilization".

In order to gain more insight into the mechanisms of energy-coupling to acid secretion in the stomach, the effects of oligomycin on respiration were studied. Oligomycin is an inhibitor of oxidative phosphorylation; (83,84); consequently, it blocks respiration coupled to phosphorylation, but has no effect on uncoupled respiration. The effects of oligomycin recorded in the literature vary depending on the laboratory. Whereas Sachs (67) observed no inhibition of acid secretion and respiration, Bannister (24) found inhibition of both parameters. Also, Durbin and Michelangeli (39) reported that in order to achieve inhibition, high levels of oligomycin and long periods of exposure were necessary. From these experiments in intact mucosa it would seem that low permeability to the compound could account for the discrepancy in the results. Isolated cells might be expected to be more permeable because fewer diffusion barriers are present.

In these experiments respiration was measured in control conditions, then oligomycin (10 μgr/ml) was added to one of two aliquots. After an incubation period with oligomycin, db-cAMP was added to both control and experimental chambers and the respiratory response was measured. At the end of one hr of exposure to db-cAMP, both aliquots were extracted with PCA in order to measure the amount of high energy phosphate compounds (cf. Methods).

TABLE V. Effect of preincubation in Cl^--free solutions on the QO_2 response to db-cAMP (5mM) in isolated oxyntic cells. See text for details.[2] (n=3; cells pooled from 4 mucosae in each experiment).

	QO_2 (µl/mg dw hr)				
SOLUTION	Control	db-cAMP		%	"p"
Cl^-	4.28 ±.21	5.75 ±.26	1.47 ±.09	34.5 ±2.4	<0.001
Cl^--free	4.94 ±.30	5.79 ±.38	.85 ±.15	17.2 ±3.3	<0.005
"p" Cl-Clfree	<0.05	n.s.	<0.005	<0.005	

Initial experiments demonstrate that exposure to oligomycin for 2.5 hr is necessary in order to completely inhibit the respiratory response to db-cAMP. Figures 7 and 8 portray typical experiments in this series. During the first hour, the addition of 10 μgr/ml oligomycin brings about a fast but small decrease in respiration. Respiration, however, comes back to control levels at the end of 2.5 hr. At this point, the addition of db-cAMP provokes an increase in respiration in the control chamber; oligomycin, however, completely annuls the increase. The subsequent addition of 0.1 mM DNP shows that oligomycin does not block uncoupled respiration. Results of five experiments of this type are shown in Table VI. As was mentioned before, there is a small but significant inhibition of respiration (13%) during the first hour of exposure to oligomycin, which reverses almost completely at the end of 2.5 hr of incubation. Addition of db-cAMP increases respiration by 33 ± 6% in the untreated aliquots, with no change whatsoever in the oligomycin-treated sample.

These results indicate that only a small amount of the basal respiration is coupled to phosphorylation in the isolated cell preparation. On the other hand, db-cAMP-stimulated respiration is entirely coupled to phosphorylation. These results are consistent with the view that ATP energizes the secretory process.

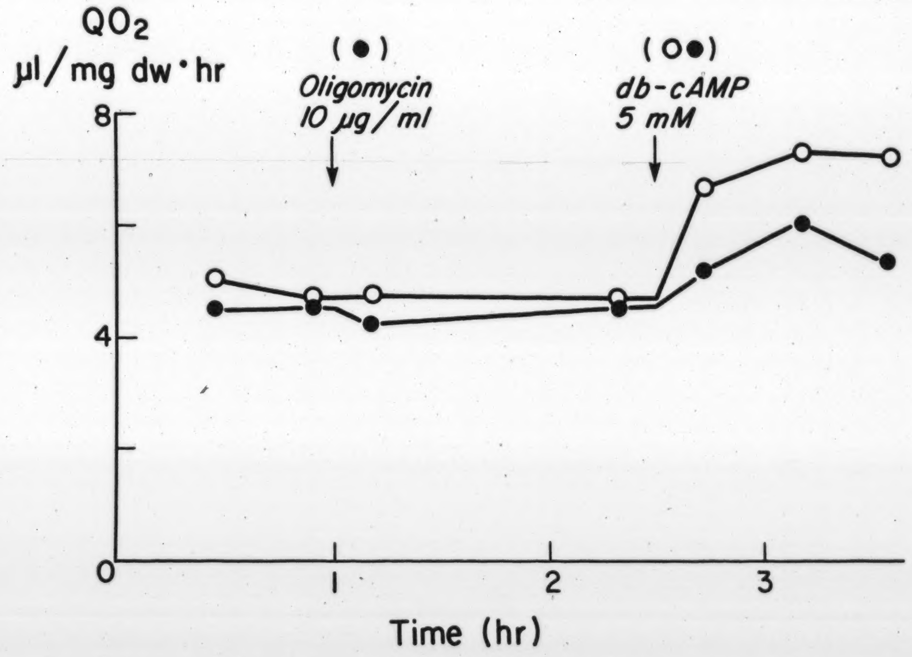

Figure 7: Effect of oligomycin (10 µg/ml) on respiration (QO_2) and the response to db-cAMP.Cells were treated with oligomycin for 1.5 hr before the addition of db-cAMP. Closed circles, oligomycin treated cells; open circles, control cells.

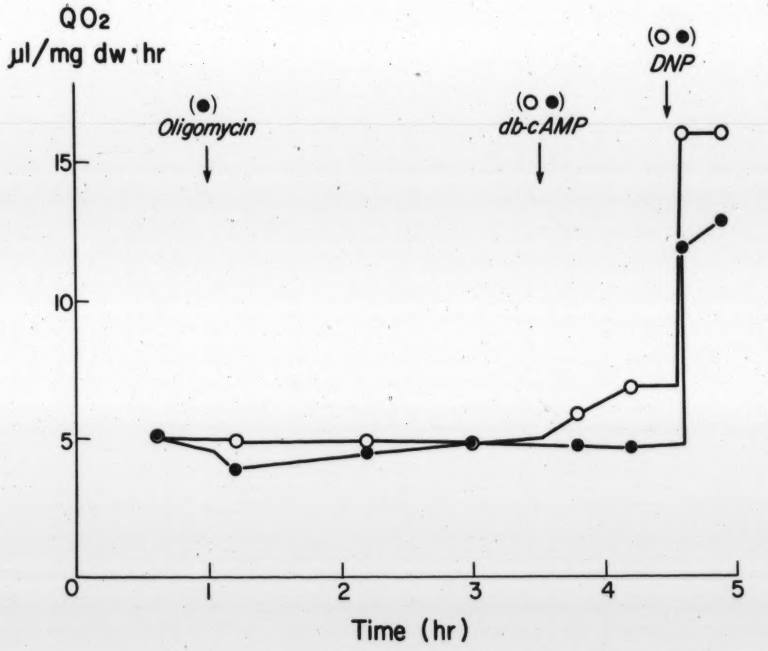

Figure 8: Effect of oligomycin (10 µg/ml) on respiration and the subsequent response to db-cAMP and DNP. Cells were treated with oligomycin (closed circles) for 2.5 hr before the addition of db-cAMP. DNP was added one hour after addition of db-cAMP.

TABLE VI. Effect of Oligomycin (10 μg/ml) on respiratory response to db-cAMP by isolated oxyntic cells (n=5; cells pooled from 6 mucosae in each experiment)

	QO_2 (μl/mg dw hr)				
	Control	1st hour	2 1/2hour	db-cAMP	%
CONTROL	4.95 ±.15	4.83 ±.09	4.75 ±.18	6.30 ±.28	33.0 ±6.0
OLIGOMYCIN	4.87 ±.17	4.24 ±.09	4.54 ±.09	4.57 ±.09	1.2 ±1.3
"p"	n.s.	<0.02	n.s.	<0.001	<0.001

C) MORPHOLOGY

Intact Tissue

The fine anatomy of the amphibian stomach at the light microscope level has been described by Norris (68). On the other hand, at the electron microscope level most reports have concentrated on the structure of the oxyntic cells. For the purpose of comparison with the isolated cells, I will briefly describe the morphological features of each type of cell.

Surface epithelial cells lining the free surface and the pits (Fig. 9) are tall, columnar cells with basal nuclei. The apical portions of the cells are occupied by membrane-bound secretory granules of variable size and irregular shape. A few small mitochondria are found scattered throughout the subgranular cytoplasm.

Mucous neck cells at the gland-pit junction (Fig. 10) are rounder and have basal nuclei. Their apical sides are packed with secretory granules of varying size, form and electron density. A few round mitochondria are seen between the nucleus and the secretory granules. Microvilli are found projecting into the lumen.

Argentaffin cells are also present (Fig. 10). These cells are arranged in groups that form a cuff around the mucous neck cells. They are also scattered all along the gland where they make contact with the oxyntic cells (Fig. 11). Argentaffin cells contain large nuclei and a large number of small membrane-bound granules. Sometimes the very dark granules may have loose fitting membranes which also have been described for the mammalian argentaffin cell (69). Most of the granules are located in the basal or infranuclear portion of the cell. Given the basal location of the granules in the argentaffin cells, and because there is no

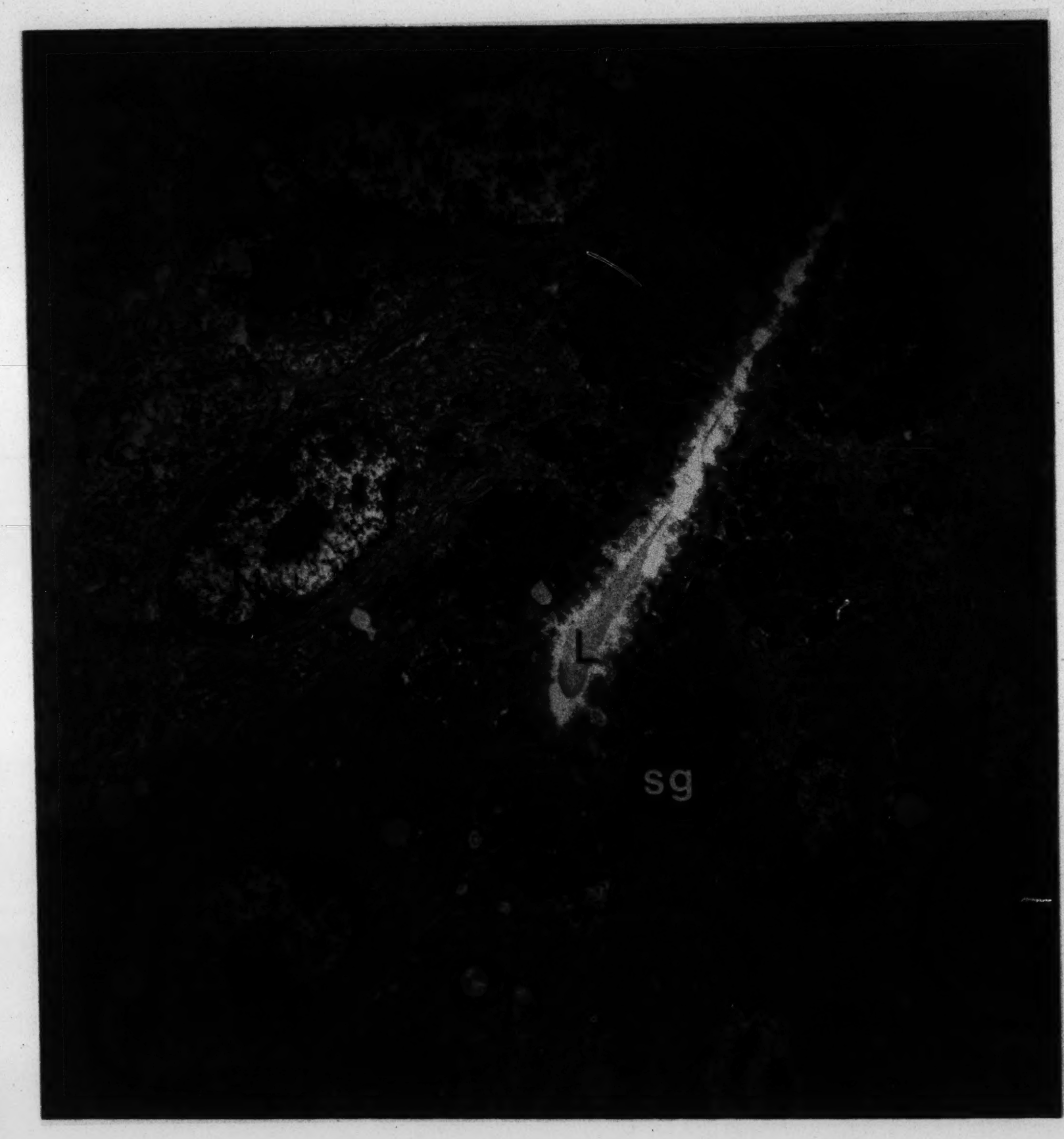

Figure 9: This micrograph shows a section of an intact gastric mucosa at the surface cell region. Surface epithelial cells line the lumen of a pit (L). The apical pole of the cell is packed with an array of secretory mucus granules (Z). Nuclei (N) are situated at the basal portion of the cells. Magnification, (6000x).

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Figure 10: Section of intact gastric mucosa through the neck region. Mucous neck cells (MNC) are seen lining the lumen of the gland. The apical pole of these cells is packed with numerous granules of different size, shape and electron density (SG). Some of these membrane-bound granules seem to make contact with the apical plasma membrane. Cells have a basal nuclei. An argentaffin cell is also seen in the micrograph, containing small dark granules most of which are in the infranuclear portion of the cell, close to the interstitial space. The basement membrane encloses both types of cells. Numerous collagen fibers are found in the interstitial space (Cg) in which a capillary (C) and several nerve endings (NE) are seen. Magnification, 9000x.

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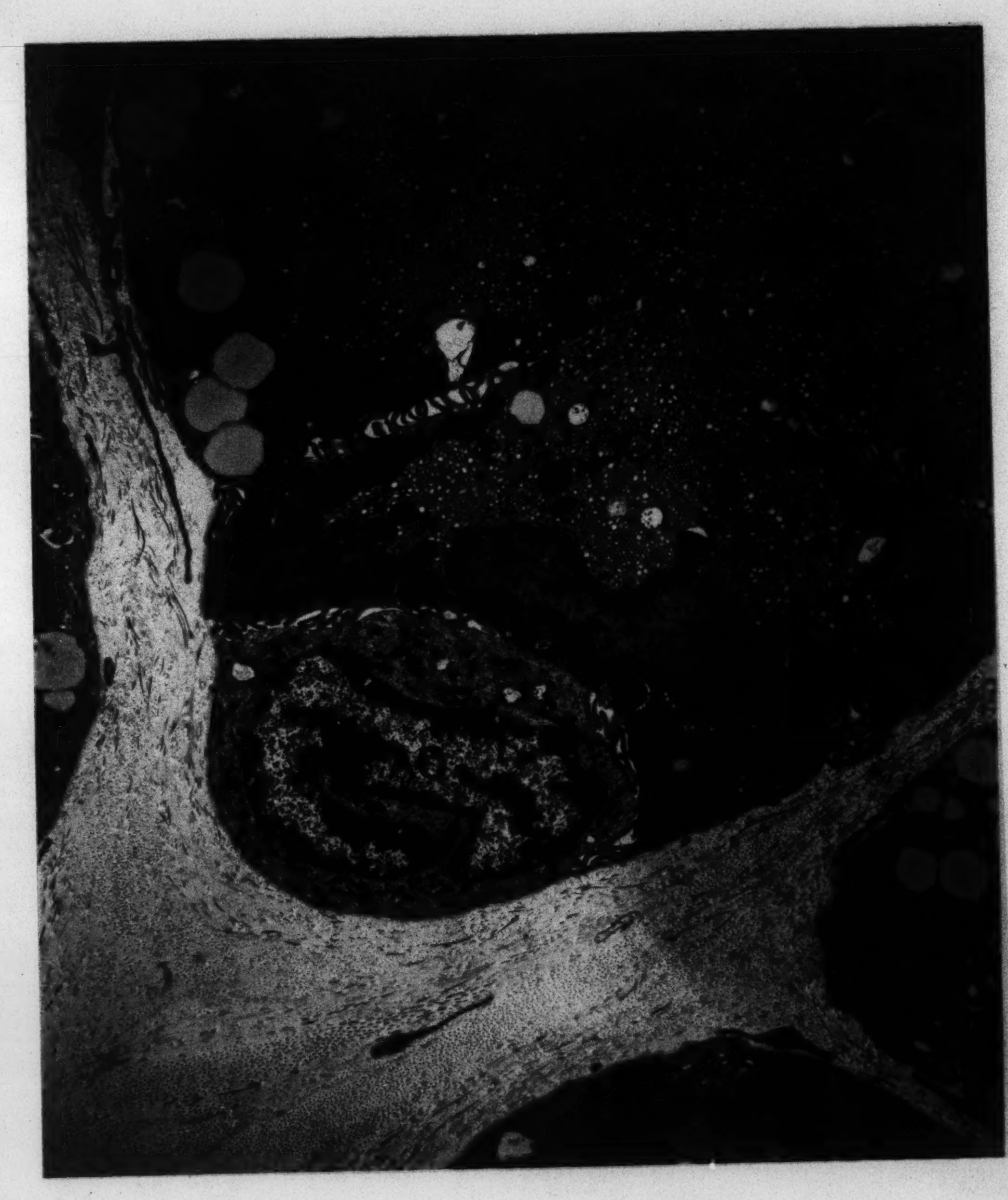
Figure 11: Section of intact gastric mucosa through the oxyntic cell region of the glands. In this micrograph an argentaffin cell is seen in close proximity to the oxyntic cell. As in the preceding figure, most of the granules in the argentaffin cell appear to be located towards the portion of the cell facing the interstitial space. Oxyntic and argentaffin cells share a common basement membrane. Oxyntic cells are described in the next figure. Magnification, 9600x.



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communication between these cells and the lumen, it might be speculated that their contents empty into the interstitial space. Furthermore, although the function of these cells is not known, they have been shown to contain histamine in both the mammalian (70) and the amphibian mucosa (71), in addition to other biogenic amines (72). Thus, these cells may represent the stores from which histamine is released by other secretagogues (60).

The structure of the oxyntic cell in the resting state in the intact tissue, and the changes induced by histamine stimulation are well documented (28-30). However, since the studies on isolated cells involved stimulation with db-cAMP, the effects of this compound on the morphology of the oxyntic cells also had to be investigated. Paired resting mucosae were obtained by means of the procedure described in Methods. One-half of the pair was stimulated with 5 mm db-cAMP and after one hr both halves were fixed.

The resting oxyntic cell displays features similar to those described elsewhere. In order to compare it with the stimulated mucosa and with isolated cells, the morphology of the resting mucosa is shown in Figure 12. This electron micrograph corresponds to a section of a gastric gland in the region of the oxyntic cells: four or five oxyntic cells border the lumen of the gland; the apical border of the cells appears smooth with a few short microvilli; and the lumen is separated from the intercellular space by functional complexes that hold adjacent cells together at the apical side. These functional complexes are formed by Zonula occuludens and a row of two or three desmosomes (73). The apical sides of the cells are packed with networks of intracellular membranous systems (T). In the literature these systems have been referred to as tubules, vesicles, tubo-

Figure 12: Electron micrograph of intact gastric mucosa; section of a gland at the oxyntic cell region. The mucosa was incubated for several hours to achieve a resting state prior to fixation. The lumen of the gland (L) is delimited by oxyntic cells. Apical border is fairly smooth with a few microvilli. T, vesico-tubular membranous system. M, mitochondria. LD, lipid droplets. N, nucleus. PG, peptic granules. Magnification (6000x)

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vesicles or vesicotubules. Lysosomes and multivesicular bodies are immersed in this network of membranes. Membrane-bound dense granules are also seen and they probably correspond to peptic granules (87). The basal part of the cell is occupied by the nucleus, abundant mitochondria and nonmembrane-bound semi-translucent granules that seem to correspond to lipid droplets (LD). Basal (serosal) membrane is rather smooth and rests on a basement membrane. The glands thus described are then immers in a mesh of collagen fibers.

A detail of the apical region of a resting oxyntic cell is shown in Figure 13. The lumen of the gland appears in the upper right corner of the micrograph. Two different cells are separated by a tortuos intercellular space that is filled with interdigitated foldings. At the apical side, the cells are held together by a typical junctional complex constituted by Zonula ocludens and three desmosomes. Some short microvilli project into the lumen. The extensive network of membranes does not seem to make any contact with the plasma membrane in these resting cells.

Figure 14 records the effect of stimulation with 5 mM db-cAMP on th morphology of intact mucosa. The lumen of the gland is filled with a striking proliferation of plasma membranes that shows up in the form o microvillous projections or long foldings filled with apical cytoplasm. In the transition from the resting to the secreting state there seems to be no other qualitative difference in regard to other intracellular organelles. By using quantitative sterological techniques, however, Helander et al. (54), and,more recently,Ito and Shofield (74) have show that the increase in plasma membrane surface is quantitatively matched b a decrease in the intracellular tubovesicular system. This proliferati

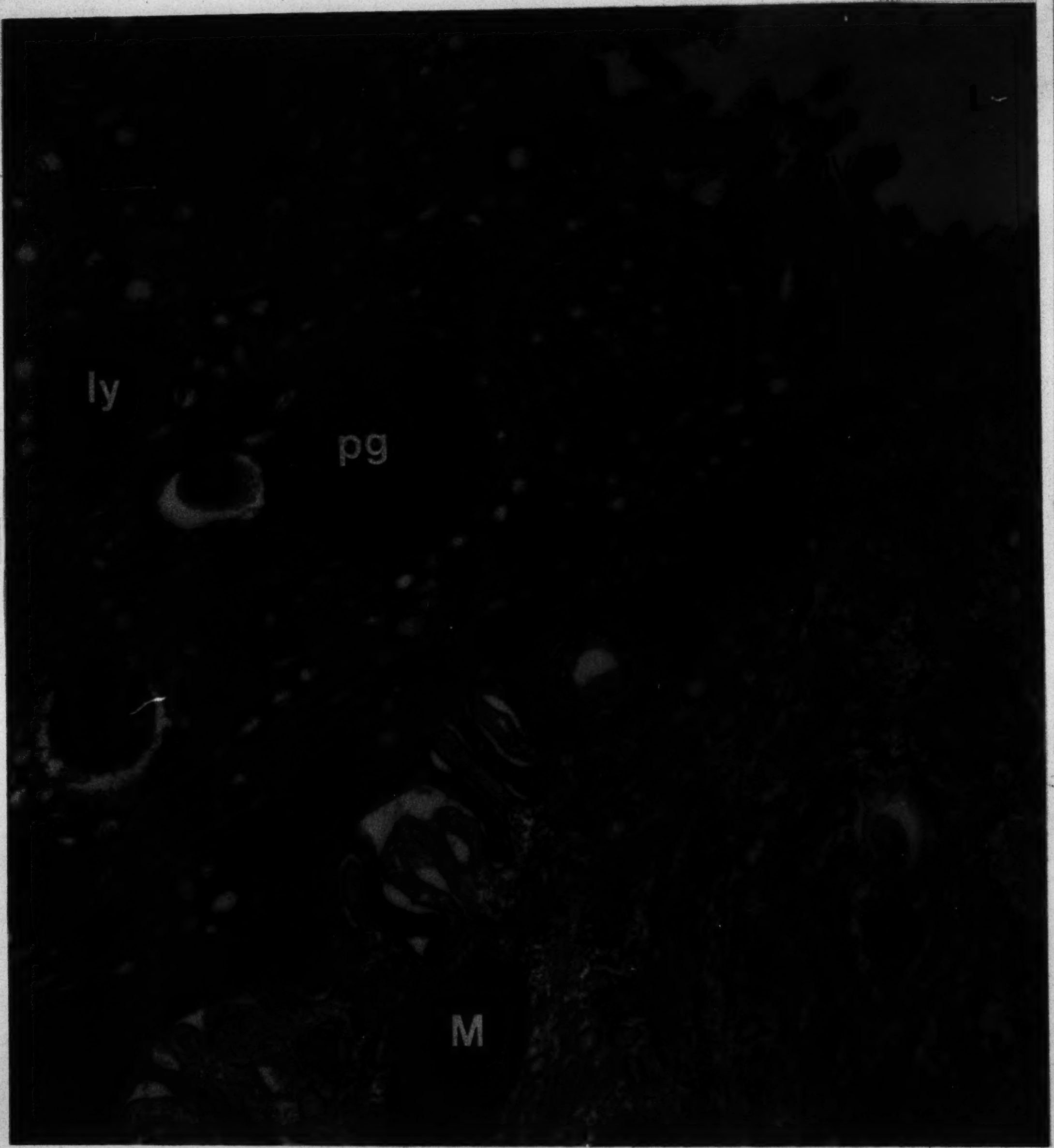

Figure 13: Portions of two adjacent oxyntic cells. This micrograph is a detail of the preceding figure. TJ, tight function. IS, intercellular space. PG, peptic granule. LY, lysosome. M, mitochondria. T, vesicotubular membranous system. L, lumen. MV, microvilli. Magnification, 42000x.

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Figure 14: Electron micrograph of intact bullfrog gastric mucosa fixed 90 minutes after stimulation with 5mM db-cAMP. The mucosa was secreting acid at a rate of 4.7 uEq/cm^2 hr at the time of fixation. Numerous projections (MV) fill the lumen of the gland at the oxyntic cell region. L, lumen; T, vesico-tubules; M, mitochondria; LD, lipid droplets; PG, secretory granules. Magnification (9000x).

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of apical plasma membrane seems to involve the fusion of the tubulovesicular system with the plasma membrane (30).

Figures 15 and 16 portray two types of microvillous projections at higher magnification: one is long and slender (Fig. 15) and seems to branch at some points; and the other (Fig. 16) is shorter, thicker, and without ramifications. These dissimilarities perhaps may be attributed to fixation because often times they do appear in distinctly different preparations. Sometimes, however, both kinds coexist in the same preparations, although this may occur because of differences in fixation in different parts of the tissue. It should be pointed out that the slender type is usually seen in regions where the lumen is ample, whereas the other shorter type has been observed in areas with lumen of smaller diameters. One might speculate that the size and shape of the microvilli depend on the diameter of the lumen. On the other hand, the degree of proliferation of apical plasma membrane may be physically limited by the size of the lumen. In both figures the arrows point to the sites where fusion of the tubulo-vesicles and plasma membrane possibly occurs. These images do not appear in the resting preparation (see Fig. 13).

Isolated Cells

At the electron microscope level quantification of types of cells was not attempted. Instead, light microscopy was utilized in the manner described above, and only for oxyntic cells. Because cells are oriented at random in the cell pellet, the plane of the section passed through diverse regions of the cells. Thus, most of the time cells were photographed when the nuclei and characteristic morphological features of a given cell-type were present in the same section; for example, secretory granules, mucous granules or mitochondria, and tubulo-vesicles. Some

Figure 15: A detail at the apical pole of an oxyntic cell in the intact mucosa actively secreting acid. Numerous slender and long microvilli and foldings project into the lumen (L). Arrows point to sites where fusion of vesico-tubules and plasma membrane possibly occurs. Double headed arrows point to sites of ramification of projections. T, vesico-tubuler membranous system. Magnification, 39000x.

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sections that showed an interesting feature were also photographed although they did not meet the above criteria.

Results from two experiments in which cells were fixed both in "resting" conditions and one hr after being stimulated with db-cAMP are presented here.

At this level, no quantification of the types of cells was attempted. Nevertheless, it was apparent that most of the sections corresponded to oxyntic cells.

Mucous-neck cells, argentaffin cells, and a very small number of surface epithelial cells are present in the non-oxyntic fraction. Some clumps of three or more cells are also present; they consist of epithelial cells, which still are attached to each other by desmosomes. This type of cell is found in the intact mucosa at the neck-pit transition and it is very similar to surface epithelial cells except that they do not contain granules.

The isolated surface epithelial cell (Fig. 17) no longer is columnar in shape, but has become round. However, some polarization still is recognizable in the sense that the secretory granules remain in one pole and the nucleus at the other. The characteristics of the secretory granules in the isolated cell are identical to those in the intact mucosa. Many microfilament bundles are seen traversing the cytoplasm of the cell. These microfilaments are seen in the intact mucosa and are most often associated with the numerous desmosomes that hold together the surface epithelial cells. Hemidesmososomes are found at different points in the walls of phagocytic vacuoles.

The isolated mucous neck cell (Fig. 18) is very similar to that in the intact mucosa. Microvilli and the relative position of the nucleus

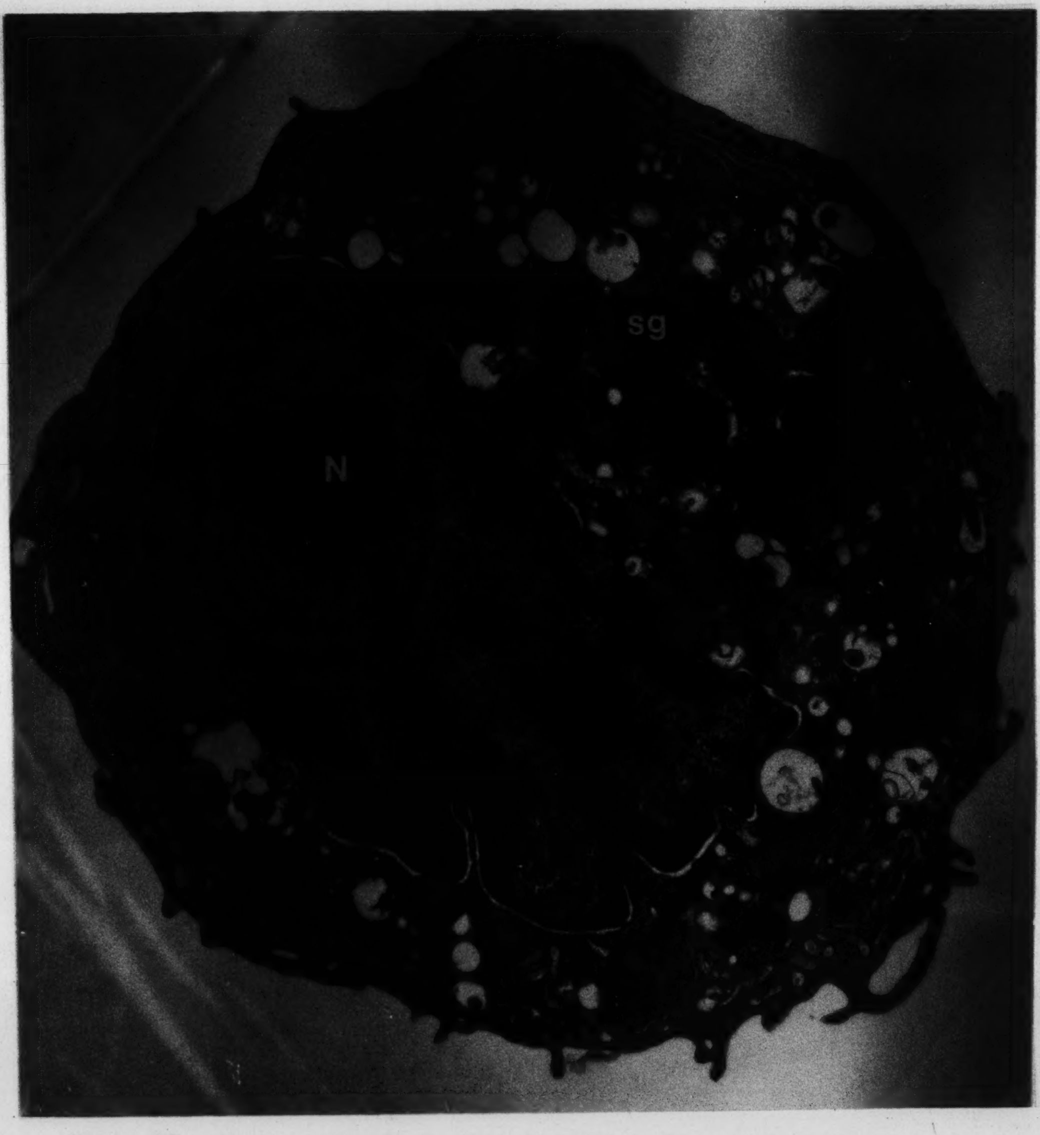

Figure 17: Electron micrograph of an isolated surface epithelial cell. Some polarization is present. Secretory granules (SG) are on one pole of the cell and the nucleus (N) at the other. Microfilament bundles (MF) traverse the cytoplasm of the cell. Hemidesmosomes (HD) are found in the walls of phagocytic vacuoles. Magnification, 18000x.

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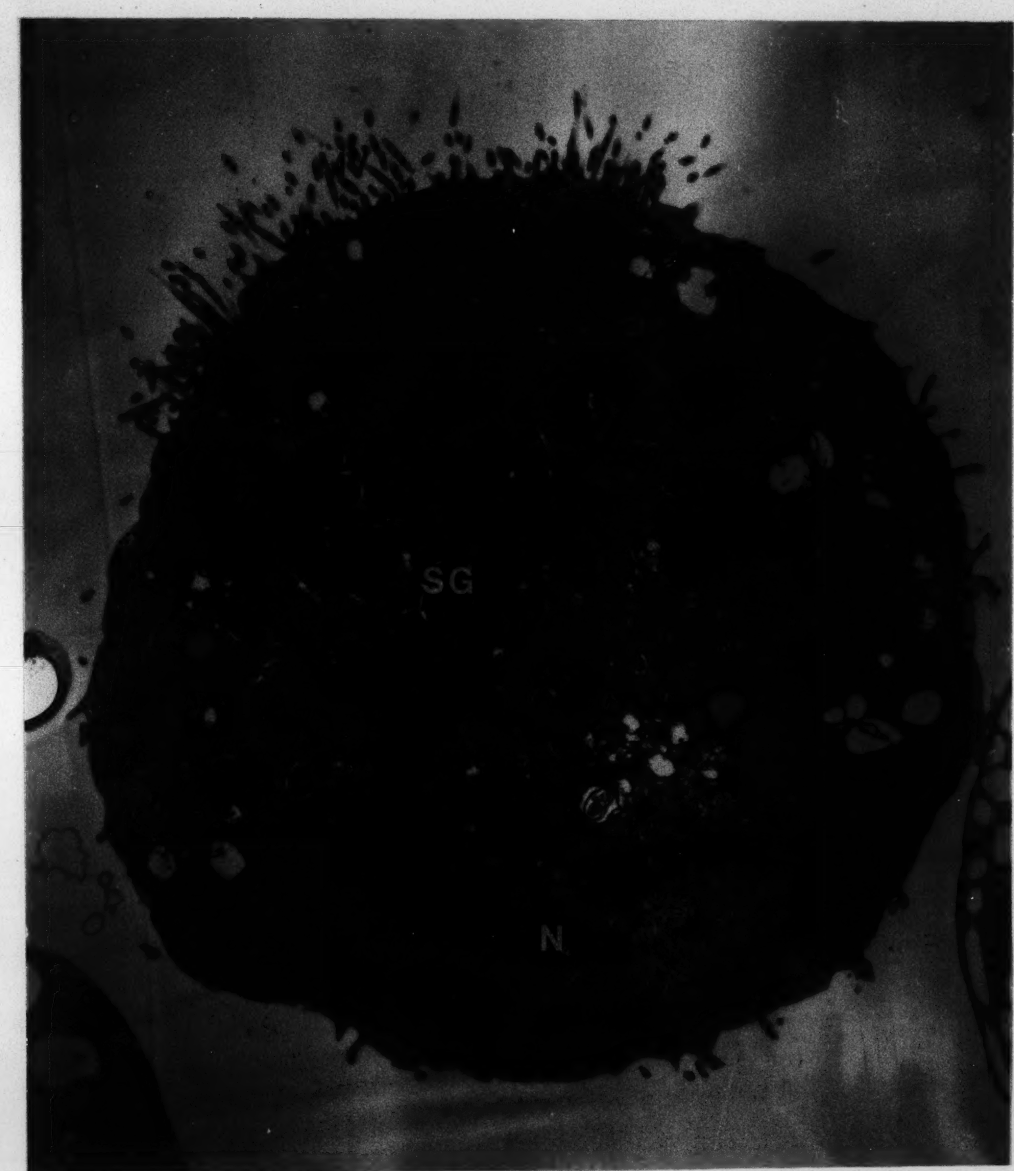

Figure 18: Electron micrograph of an isolated mucous neck cell. A large degree of polarization is recognizable. Numerous secretory granules (SG) occupy most of the cytoplasm of the cell. The presence of microvilli mark the site corresponding to the apical plasma membrane. Magnification, 13000x.

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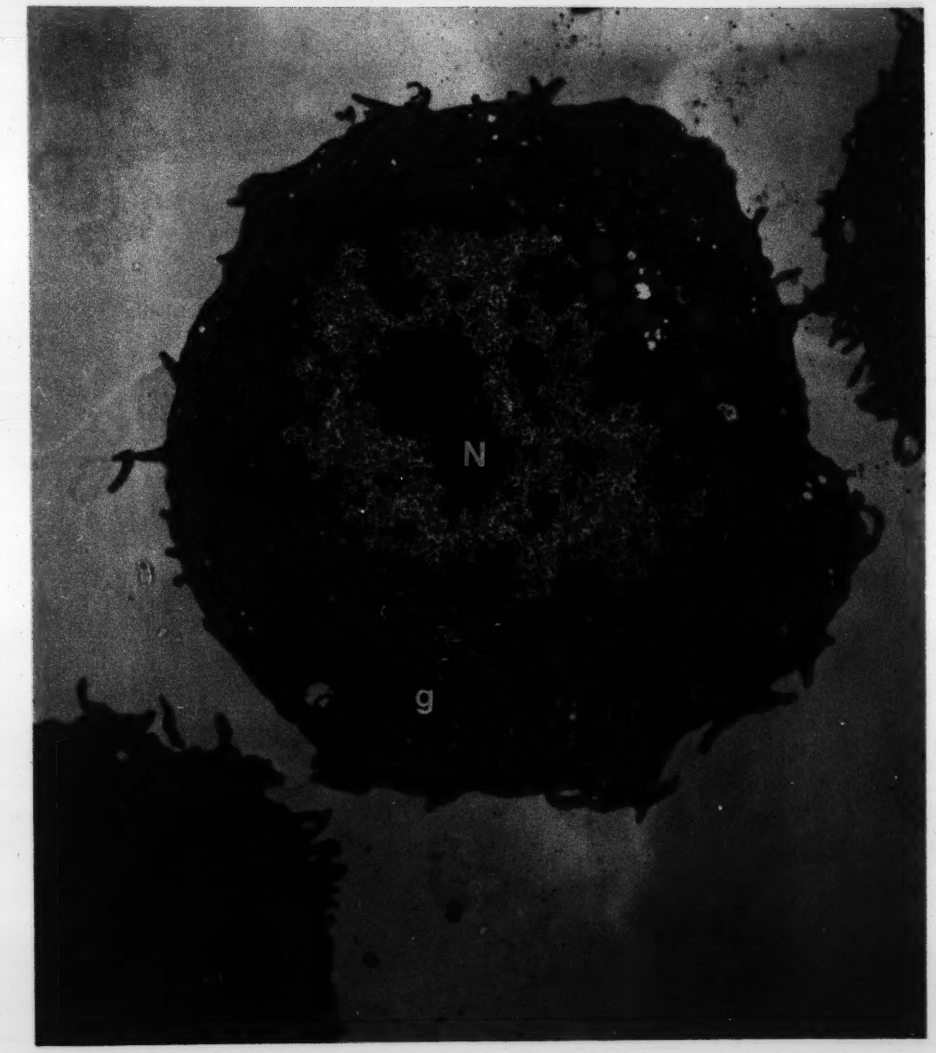


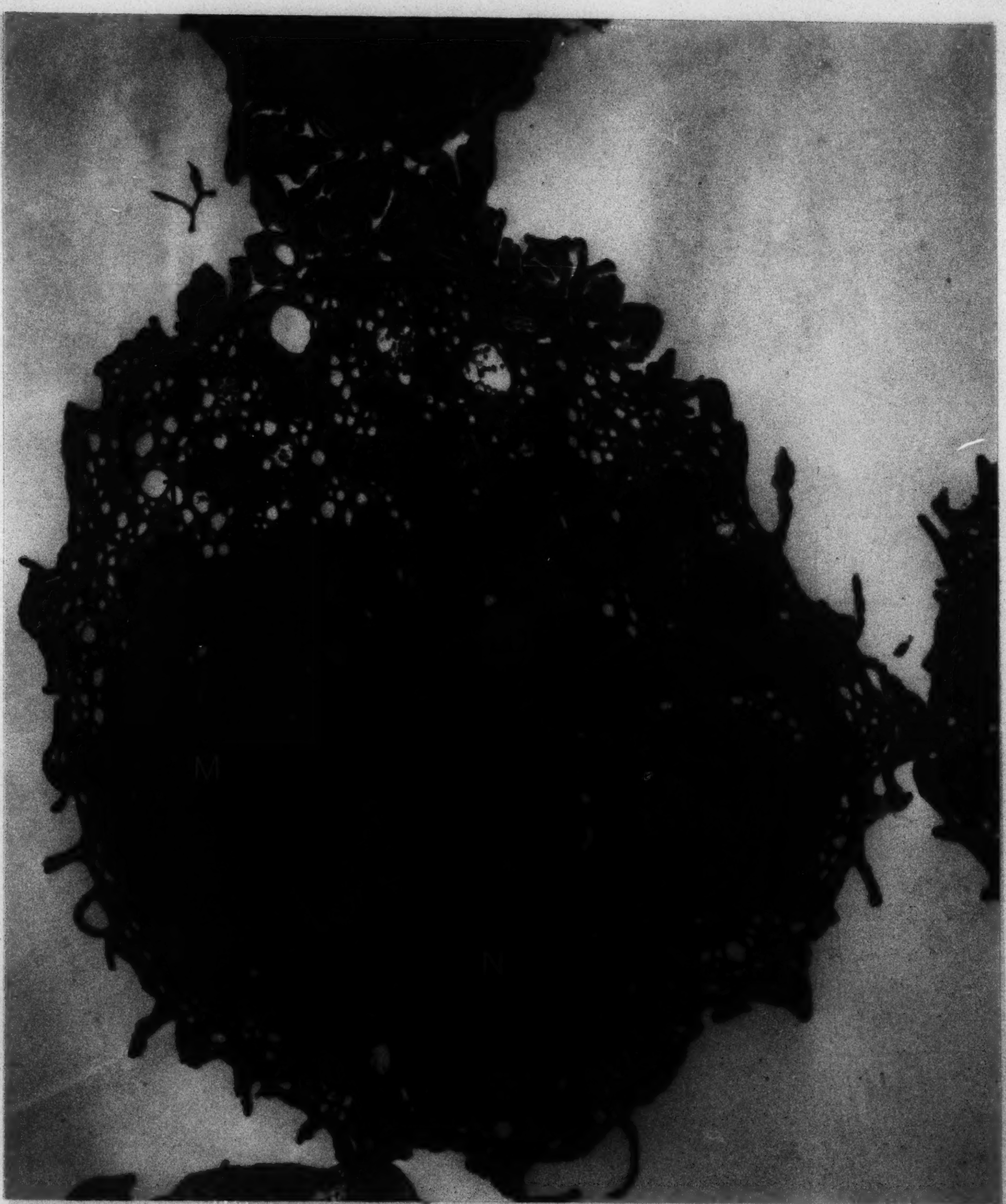
Figure 19: Electron micrograph of an isolated argentaffin cell. Although no polarization can be recognized, it is seen that most of the granules (G) are situated on one side of the cell. Compare to figures 10 and 11. N, nucleus. Magnification, 16000x.

and granules indicates that polarization still exists after isolation. These cells appear to contain many more granules than those in the intact mucosa; they also seem to have grown in size because of an increase in the number of secretory granules (compare to those in Figure 10). This may reflect a lack of normal stimuli for granule discharge in the isolated cell preparation.

Figure 19 shows an argentaffin cell after isolation from the mucosa. Although it is more difficult to ascertain polarization in these cells, the granules are facing the interstitial space located mostly on one side of the cell. Essentially the number of granules does not differ qualitatively from that observed in argentaffin cells in the intact tissue.

The isolated resting oxyntic cell Since this was a limited study and because of the problems involved in applying quantitative stereological techniques to the isolated cells (vide infra), several different sections of isolated oxyntic cells are shown both in the resting and stimulated state.

Figures 20 to 23 show different examples of resting isolated oxyntic cells. Although the cell is no longer oriented in the frame of the epithelium, a large degree of polarization is recognizable. The relationship between nucleus, mitochondria, and vesico-tubules, resembles that in the intact mucosa. In a large percentage of the observed cells, the surface membrane is fairly smooth with a few microvillous projections: Some of these projections have the characteristics of interdigitating foldings that fill the intercellular space. In some cells, such as the one shown in Figure 23, a larger number of microvilli and foldings can be seen; in this case it is difficult to distinguish proliferation at the apical side from foldings at the basolateral surface.



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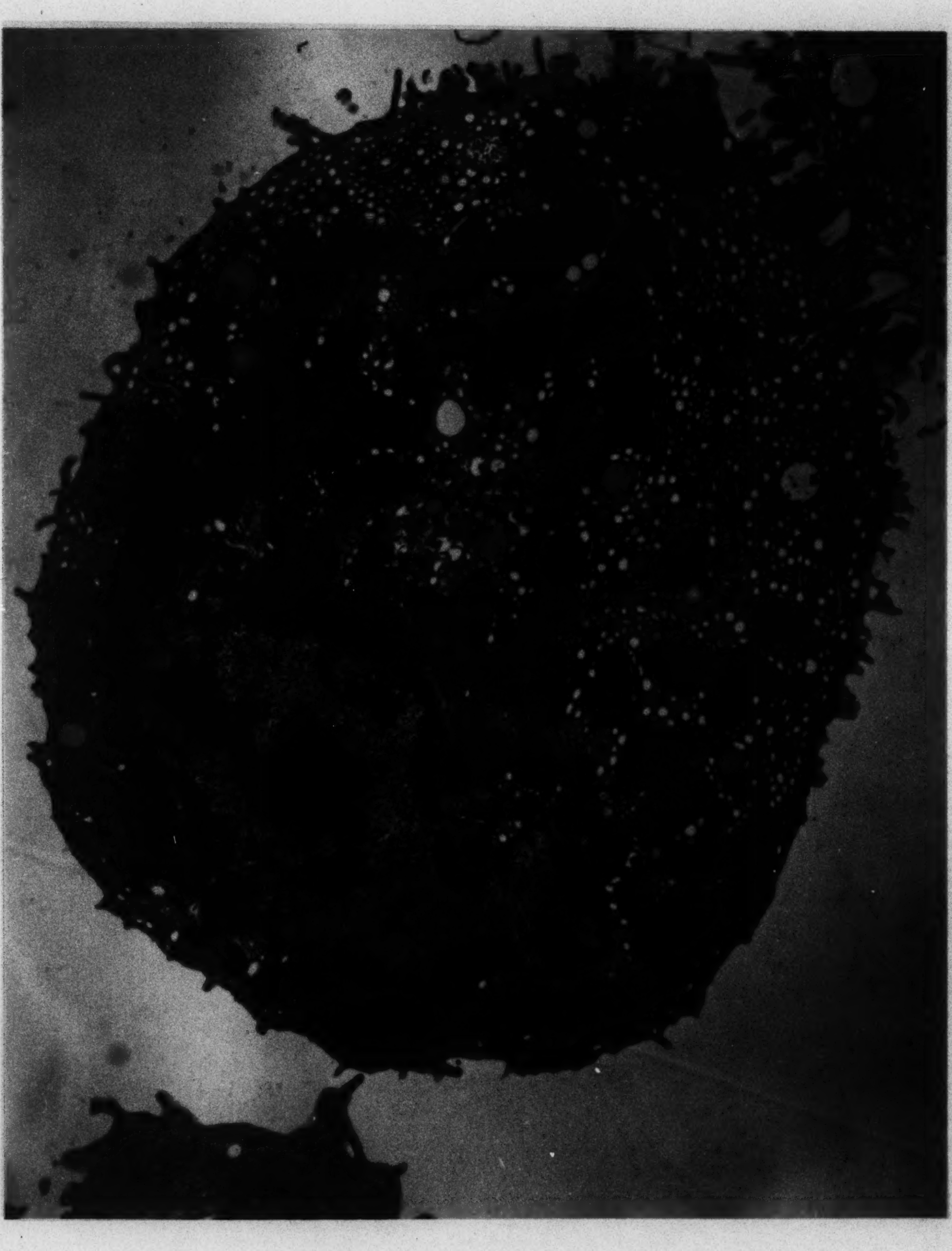

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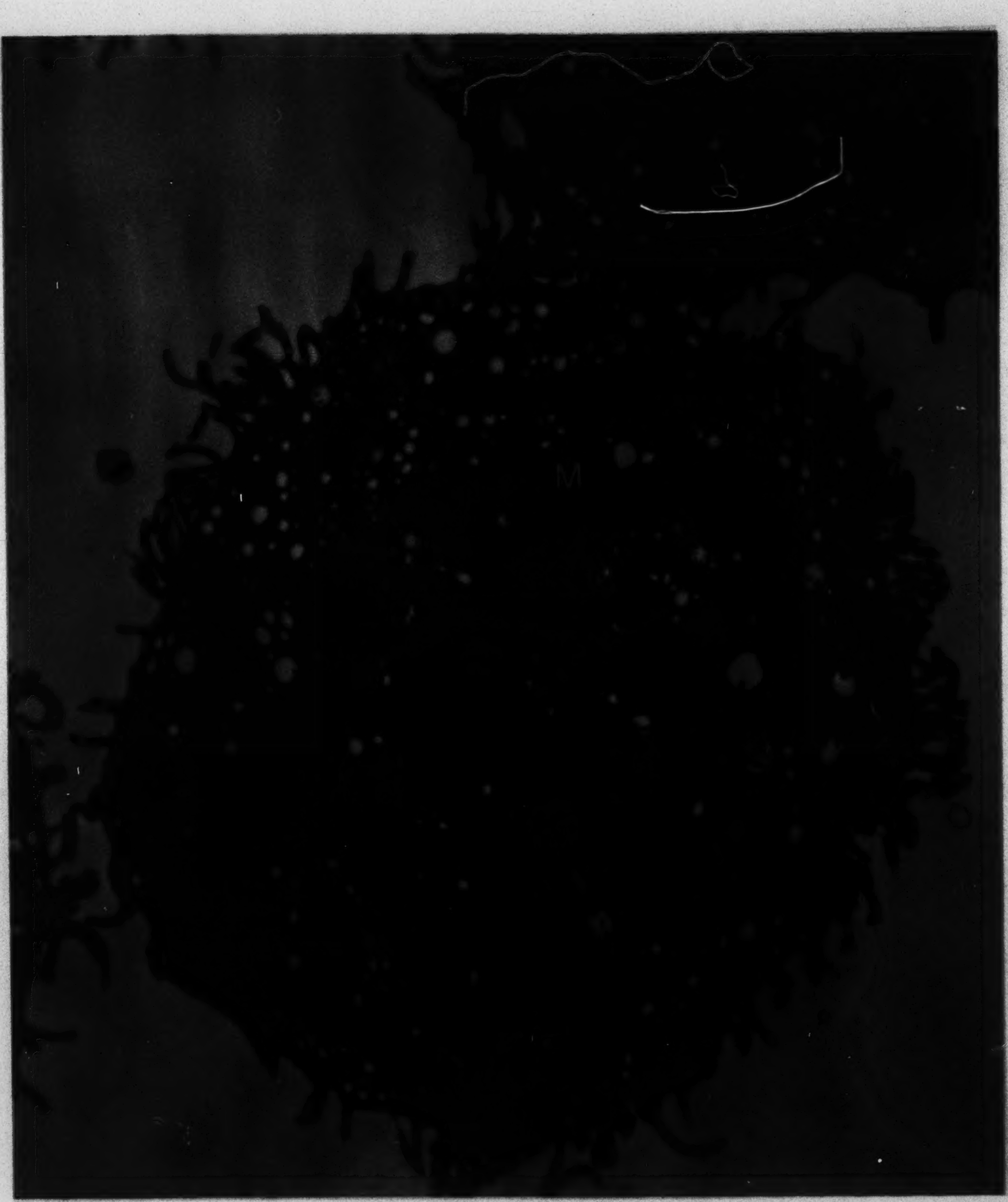

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The tubulo-vesicular system occupies a large proportion of the cytoplasm in the resting cell. As is the case in the intact mucosa, this extensive network of membranes does not seem to make contact with the plasma membrane (Fig. 24).

Other organelles-like nucleus, mitochondria, lipid droplets, and secretory granules do not show any qualitative difference in comparison with intact mucosa.

The stimulated oxyntic cell Stimulation with 5 mM db-cAMP produces dramatic changes in the structure of isolated oxyntic cells. Different examples are shown in Figures 25 to 28. A striking proliferation of plasma membrane appears as long microvilli, lingulae and foldings, of which many are confluent or ramified. In some cases they form a very intricate net.

Changes in structure often seem to occur over the entire surface of the cell. Recognizing the apical or basolateral poles of the cells is difficult: in the example shown in Figure 26, the apical pole can be identified by the presence of two hemidesmosomes still attached to the plasma membrane (indicated by arrows). Although the area between the two hemidesmosomes seems to have undergone a greater structural change, proliferation of plasma membrane can be seen outside the area delimited by the hemidesmosomes. Possibly plasma membrane newly formed at the "apical" region of the cell then spreads over the entire surface of the cell.

By now the tubulo-vesicular system has almost disappeared. Only a few vesicles remain in the cytoplasm, which may be an indication of the complete transformation of tubulo-vesicles into new plasma membrane.

In some cells, changes in the surface are less marked (Fig. 27),

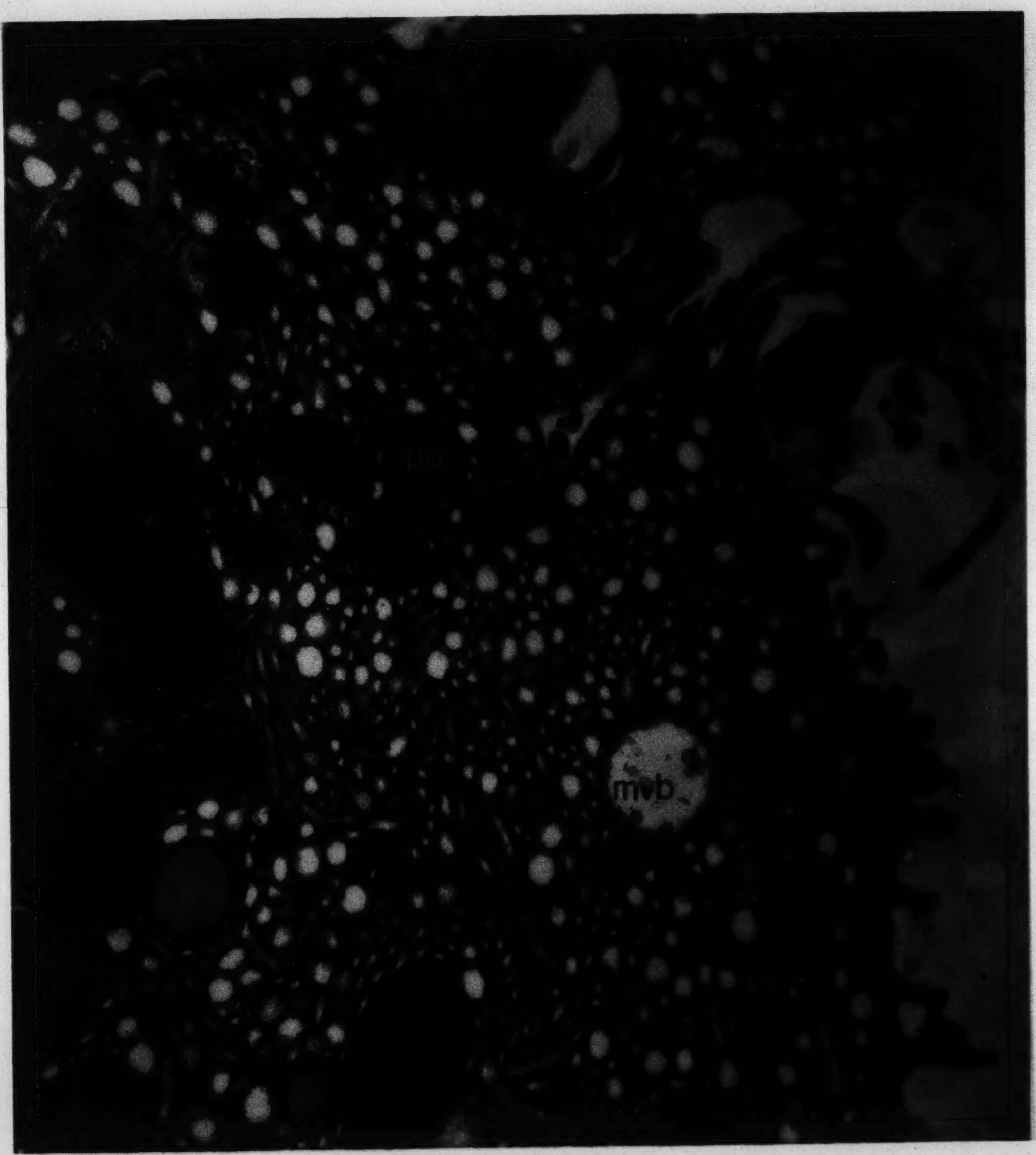

Figure 24: Detail of an isolated oxyntic cell in the resting state. As seen in the resting oxyntic cell in the intact mucosa, the vesicotubular system (T) does not make contact with the plasma membrane. M, mitochondria. C, centrosome. MVB, multivesicular body. PG, peptic granule. Magnification

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Figures 25-28: These electron micrographs portray four different examples of isolated oxyntic cells fixed 60 minutes after stimulation with 5 mM db-cAMP. Numerous microvilli and foldings appear in the surface. A few vesicles (V) remain in the cytoplasm. In Figure 26, two hemidesmosomes still attached to the surface (indicated by arrows) suggest that the area comprised between them corresponds to the apical plasma membrane. In Fig. 27, a lesser degree of membrane proliferation is accompanied by the presence of a larger number of vesico-tubular structures (T). N, nucleus. M, mitochondria. L.D., lipid droplets. Magnifications: Figure 25, 15,000x ; Figure 26, 16,000x ; Figure 27, 12,000x ; Figure 28, 13,000x.

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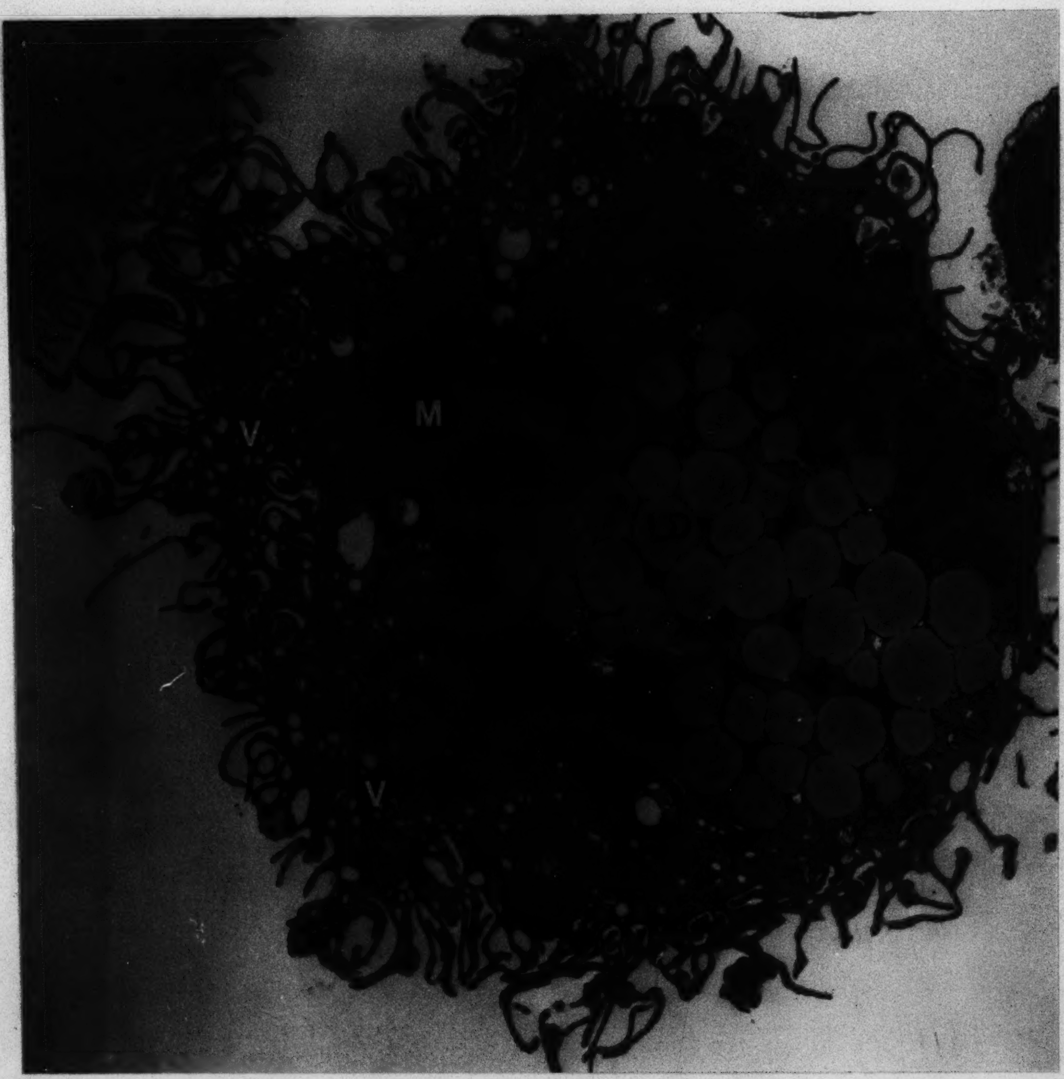
V
M
V

and are accompanied by the remaining in the cytoplasm of a larger number of tubulo-vesicular profiles. Thus, this cell may have been stimulated to a lesser extent.

The presence of a larger number of multivesicular bodies in the stimulated cells also was observed. Their function, however, of these organelles, normally occurring the oxyntic cells is still obscure. It has been suggested (74) that they may participate in the normal turnover of the plasma membrane. In this regard, it is interesting to point out that in isolated cells hemidesmosomes are found in the walls of multivesicular bodies (Figure 29).

p-Nitro-phenyl phosphatase

The fact that the cells have a random orientation and the difficulty of ascertaining the direction of the plane of section, makes the application of quantitative sterological techniques a difficult task. Consequently, another method was employed to follow the changes in surface area. Durbin and Kircher (51) have reported surface phosphatase to be present in the gastric mucosa. This ectoenzyme hydrolyzes p-nitrophenylphosphate when it is extant in the secretory solution that bathes the mucosa in the Ussing chamber. The same researchers found a correlation between acid secretion and enzyme activity under a variety of conditions, such as stimulation by histamine, and inhibition by anoxia. Changes in enzyme activity correlate at least qualitatively with predicted changes in surface area, thus, they suggest that the phosphatase may serve as a dynamic marker for the apical plasma membrane.

In this study, I applied the above findings to the isolated cell preparation. Figure 30 shows one experiment of a series in which pNPPase activity and oxygen consumption were measured simultaneously in isolated

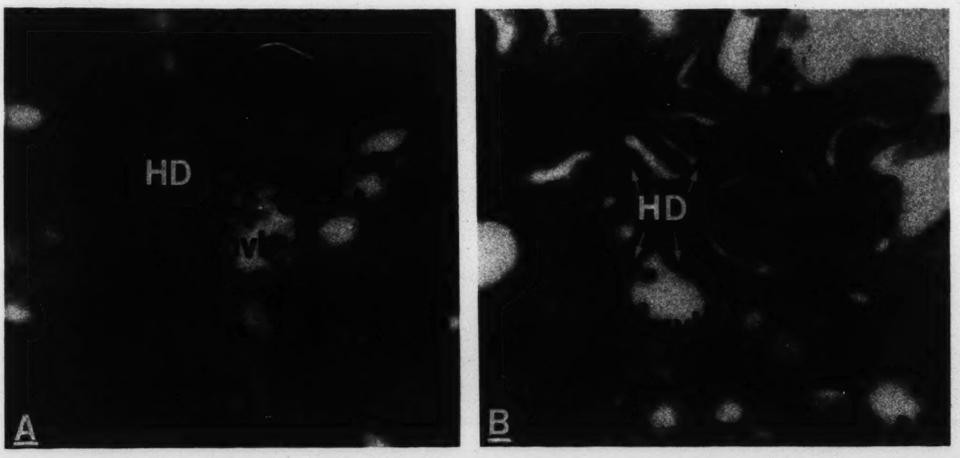

Figure 29: Detail of multivesicular bodies in isolated oxyntic cells. Hemidesmosomes are seen attached to the walls of MVB. In upper part Fig. 29B a section of plasma membrane with two hemidesmosomes is seen invaginating. Magnification 45,000x.

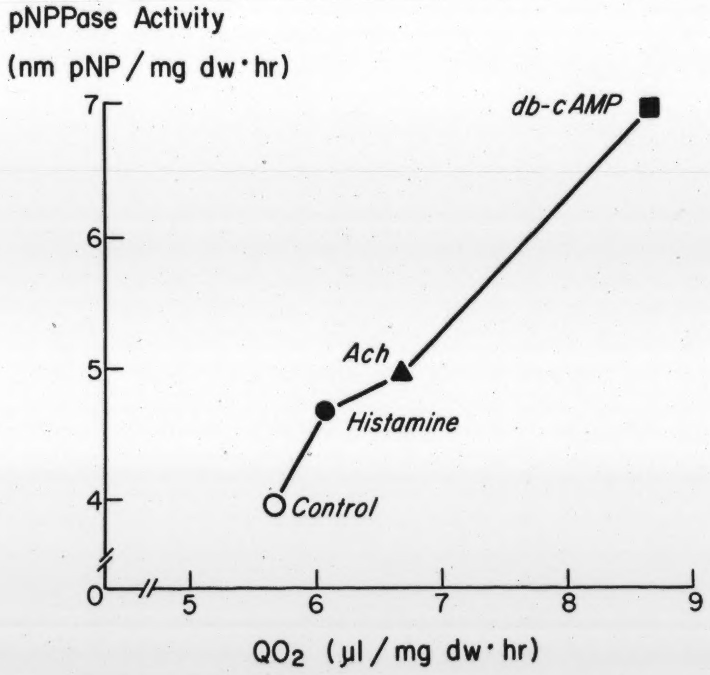

Figure 30: Effect of secretagogues on p-nitrophenyl phosphatase activity (pnPPase) and oxygen consumption (QO_2) in isolated oxyntic cells. Parameters were measured simultaneously in control conditions (0) and during stimulation by histamine (●), acetylcholine (▲) and db-cAMP (■).

oxyntic cells. These parameters were measured in control conditions and then stimulated with histamine (10^{-4}M), acetylcholine (10^{-3}M) and db-cAMP (5x 10^{-3}M). These secretagogues increased activity of the ectoenzyme from control conditions, as well as increasing oxygen consumption as was shown earlier in this study. Changes in respiration and phosphatase activity closely correlate in all experiments.

Mean phosphatase activity is 3.8 nMoles Pi/mg dw hr. This value is in the same order of magnitude as that found by Durbin and Kircher. Changes induced by secretagogues include: histamine, 21.1%; acetylcholine 28.2%; and db-cAMP, 65.8%; and they are accompanied by increases in respiration of 7.5%,11.7%and 38.5% respectively (Table VII). The preceding results demonstrate a close interaction between metabolic and structural modifications that take place upon stimulation.

D) High Energy Phosphate Compounds

In many tissues maintenance of normal concentrations of adenine nucleotides is necessary for the transport processes. Interference in the gastric mucosa with the normal mechanisms of synthesis and the maintenance of ATP levels leads to inhibition of acid secretion (39,75,76). Furthermore, stimulation of the secretory process alters the concentrations of some nucleotides and their ratios, which to a large extent depend on the activity of the pump (39,40). Thus, it is important to know whether the isolated oxyntic cells are capable of maintaining normal concentrations of high energy phosphate compounds, since this would indicate an intact metabolic machinery. Also, the ratio of nucleotides may provide a reliable index of secretory activity.

1) Intact mucosa

Since the results reported by Durbin et al. (39,40) involved stimu-

TABLE VII. Effect of secretagogues on pnPPase activity and respiration in isolated oxyntic cells. Both parameter measured simultaneously. (n=3; pooled cells of 4 mucosae in each experiment). Values are means ± S.E.

	p-nPPase		QO_2	
	(μMoles p-nP/mg dw hr.)	(treated vs Control)	(μl/mg dw hr.)	(treated vs control)
CONTROL	3.8 ± .4	-	5.3 ± hr	-
HISTAMINE	4.59 ± .43	21.1 ± 2.2	5.71 ± .62	7.5 ± 1.5
Ach	4.91 ± .70	28.2 ± 4.6	5.92 ± .60	11.7 ± 3.5
db-cAMP	6.25 ± .50	65.8 ± 9.8	7.41 ± 1.04	38.5 ± 8.3

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lation with histamine, it was necessary to study the effects of db-cAMP on the adenine nucleotide levels in the intact mucosa. High energy phosphate compounds were measured in resting conditions in paired halves of mucosae and stimulated with 5 mM db-cAMP (Table VIII).

Stimulation with db-cAMP produces effects similar to those found by Durbin et al. (40) in histamine-stimulated mucosae. Db-cAMP increases acid secretion from a resting level of 1.5 µEq/hr to 8.6 µEq/hr. ATP levels are not changed by stimulation as has been reported (40,49,76), whereas ADP is increased, resulting in a drop in the ATP/ADP ratio. In addition, AMP concentrations are also raised significantly with a concomitant decrease in the ATP/AMP ratio.

Changes in adenine nucleotides are accompanied by an increase in phosphocreatine (PCr) levels. The PCr/Cr ratio is increased in every experiment of this series. However, because of the small number of experiments and the scatter, it does not reach the level of significance.

In essence these results are similar to those found by Durbin et al. for histamine-stimulated mucosae (40). In addition the finding of a decrease in ATP/AMP ratio upon stimulation, as well as the decrease in ATP/ADP ratio indicates an increase in ATP utilization in the secreting mucosa, which when linked to the known effects in respiration suggests a concomitant increase in phosphorylation. Explanations have been offered for the reason PCr/Cr changes in the opposite direction (40), and they include shifts in intracellular pH, the effects of secretagogue on different nucleotide pools, and substrate mobilization.

Results in this series of experiments demonstrate that db-cAMP produces effects identical to histamine on adenine nucleotides, which may suggest that the effects of histamine are mediated through an increase in

TABLE VIII. Effect of db-cAMP on acid secretion (S.R.) and adenine nucleotides in intact mucosa. Paired mucosa were incubated without dogerous secretagogues until both secretory rates dropped to a resting level (Cf Methods). One of each pair was then stimulated with 5m of db-cAMP for 1 hr. Four paired experiments. Values are means ± S.E. SR, Secretory rate. NS, non significant.

	ATP	ADP	AMP	PCr	Cr	ATP/ADP	ATP/AMP	Pcr/Cr	SR
	(nanoMoles/mg wet weight)								µEq/hr.
Control	1077 ±138	145.8 ±19	10.7 ±4.7	957 ±108	740.0 ±49.0	7.47 ±.48	109.7 ±24.8	1.29 ±.09	1.5 ±.27
Stimulated	1221 ±95	218.5 ±16	26.7 ±5.9	1257 ±151	790.8 ±84.5	5.61 ±.38	51.4 ±9.0	1.60 ±.16	8.6 ±.67
"p"	n.s.	<0.005	<0.05	<0.05	n.s.	<0.005	n.s.	<0.05	<0.001

the intracellular cAMP levels.

2) Isolated Cells

Since the results of experiments in isolated cells are expressed in **nMoles/mg** dry weight of cell, a conversion factor of three can be used to obtain the wet weight of the cells. Conversely, in order to compare them with intact mucosa, the values obtained in isolated cells can be divided by three--a figure obtained from experiments where the wet/dry ratio of cells was measured (see Intracellular pH) (50)

(a) Effects of stimulation

The effects of stimulation on the concentrations of high energy phosphate compounds were studied in isolated oxyntic cells. Primarily db-cAMP was used, although some experiments also employed histamine and acetylcholine.

Initially the effects varied from day to day. The results, however, can be separated into two different groups; they are presented in Table IX. Table IXa displays the results in which effects were obtained and Table IXb concerns those experiments with little or no effects.

Concentrations of high energy phosphate in isolated cells are almost three times higher than those found in intact tissue. This is not surprising given the high proportion of metabolically active cells. On the other hand, extracellular space is not taken into account in calculating the intracellular concentration. Nevertheless, the results indicate that isolated oxyntic cells are able to maintain normal concentrations of ATP and phosphocreatine. Furthermore, the relationship between phosphocreatine and ATP (PCr > ATP) suggests the presence of an intact metabolic machinery. The first sign of metabolic inhibition is marked by a fall in PCr below the level of ATP (39,45).

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TABLE IX. Effect of db-cAMP (5mM) on adenine nucleotides in Isolated oxyntic cells. A. n=3 B. n=4. (pooled cells from 6 mucosae in each experiment). See text for details.

A

	ATP	ADP	AMP	PCr	Cr	ATP/ADP	ATP/AMP	PCr/Cr
	(nMoles/mg dw)							
CONTROL	11.197	1.146	.043	13.988	4.796	9.79	260.4	3.00
db-cAMP 5mM	10.706	1.439	.282	12.621	7.299	7.70	38.0	1.95

B

	ATP	ADP	AMP	PCr	Cr	ATP/ADP	ATP/AMP	PCr/Cr
	(nMoles/mg dw)							
CONTROL	8.130	1.329	.133	10.020	5.297	6.23	68.0	2.07
db-cAMP 5mM	8.754	1.451	.137	10.393	6.194	6.14	68.6	1.75

Stimulation with db-cAMP produces a decrease in ATP levels accompanied by an increase in ADP and AMP levels (see Table IXa), and a resultant decrease in both the ATP/ADP and ATP/AMP ratios. The effects of db-cAMP on adenine nucleotides are similar to those produced in the intact mucosa. The decrease in the ATP/AMP ratio is quite pronounced in this set of experiments and indicates that the adenylate kinase reaction plays a significant role in this tissue. Phosphocreatine and creatine behave differently in isolated cells than in intact mucosa: a decrease in phosphocreatine is accompanied by an increase in creatine, resulting in a decrease in the PCr/Cr ratio, which is contrary to what could have been expected. The results in isolated cells are more consistent with an overall increase in energy requirements induced by stimulation. This difference may be due to the high content of oxyntic cells in this preparation: oxyntic cells may respond differently from other types of cells present in the tissue.

Experiments using histamine and acetylcholine as stimulants (the results are not given in Table IX) give essentially similar results. Adenine nucleotides and the phosphocreatine/creatine couple are affected in the same manner as has been observed with db-cAMP. The magnitude of these effects is different for each secretagogue. Nevertheless, the order of potency for the depression of all ratios is: histamine > acetylcholine db-cAMP. These results are unexpected since the order of potency in stimulating QO_2 is the reverse. Their probable significance will be considered later.

Results in this set of experiments indicate that isolated cells can maintain normal concentrations of these compounds and that their ratios, at least for adenine nucleotides, change upon stimulation in a manner similar to that of the intact mucosa.

In experiments presented in Table IXb, little or no effect was found upon stimulation. A possible interpretation is that the cells are damaged somehow and secretagogues are unable to produce the expected changes. However, important differences can be observed in comparison with the results in Table II. The values found for nucleotides and ratios in all four conditions (control, histamine, acetylcholine and db-cAMP) resemble those for stimulated cells, rather than for the controls. This fact may imply that at the beginning of the experiment these cells are in a secreting or stimulated state. Consequently, secretagogues produce little or no effect. An alternative explanation is that stimulants may have multiple and even opposite effects in some preparations: i.e., the secretagogue may induce a decrease in ATP/ADP and PCr/Cr ratios by stimulating the pump, and in addition increase glycolysis and lipolysis, which would tend to increase the preceding ratios.

In order to explore these possibilities and answer these questions, the original protocol was modified as follows.

Cells were suspended in 30 ml of 40 mM TES-Nutrient without K^+ with 10% albumin and kept at 4^oC for three hr after which they were centrifuged and suspended again in the same solution at an approximate concentration of 0.5 mgr/ml. Aliquots of 5.2 ml were used in each chamber. The addition of 5 mM K^+, db-cAMP, and histamine were made directly to the chamber. Five ml of the cell suspension were centrifuged at the end of the incubation period in order to extract the pellet. All other steps were performed as described in Methods. Oxygen consumption was measured simultaneously.

The rationale for this experiment is that if cells are stimulated by the presence of large amounts of histamine that are released possibly from the extant argentaffin cells, the use of a smaller concentration of cells and a larger volume would dilute the compound so that its concentration would be below threshold levels. On the other hand, withdrawal of K^+ from the solution, would inhibit any secretory activity by the isolated cells, as has been shown to occur in the intact mucosa (77). Potassium-ion withdrawal was chosen as an inhibitor because of the convenience in readding it when necessary. Furthermore, inhibition by these means ~~render~~ renders a preparation that has the characteristics of the resting stomach at the morphological level (78). In addition Durbin et al. (40) have shown that the absence of K^+ gives adenine nucleotide ratios typical of resting stomachs. On the other hand, the levels of these compounds are absolutely normal, suggesting that K^+ is not essential for the production of energy. Thus, K^+ may be needed only at the pump site and for the functioning of an ATPase, as has been postulated by Ganser and Forte (79,80) and by Scholes (81). These characteristics of the inhibition of the secretory process by K^+ withdrawal make it a suitable tool for this experiment.

The effects of K^+ withdrawal on high energy phosphate compounds and respiration are shown in Table X. Four aliquots are studied in each experiment: one without K^+; one without K^+, but with db-cAMP; one with 4 mM K^+; and one with 4 mM K^+ and db-cAMP. This table shows that the concentrations of adenine nucleotides in K^+-free solutions are normal. Furthermore, the PC/ATP ratio is rather high (PCr > ATP) indicating that under these conditions isolated cells metabolize normally. ATP/ADP, ATP/AMP and PCr/Cr ratios show values similar to those obtained in the resting state. The rate of respiration is relatively slow in these conditions.

TABLE X. Effect of db-cAMP (5mM) and K^+ readdition on adenine nucleotides and respiration in K^+-depleted isolated oxyntic cells. Isolated oxyntic cells were incubated for 3 h at 2°C in K^+-free media. Cells were then warmed up to 25°C and divided into 4 aliquots. After 15 minutes additions were made. QO_2 was measured 40 minutes after additions. Cells extracted for the measurement of metabolites 1 h after addition. Numbers in parentheses under QO_2 values represent % stimulation in relation to K^+-free. Average of 3 experiments (cells pooled from 6 mucosae in each experiment).

	ATP	ADP	AMP	PCr	Cr	ATP/ADP	ATP/AMP	PCr/Cr	QO_2
	(μMoles/gr dw)								(μl/mg dw hr)
K^+-free	12.687	1.685	.046	16.544	5.854	7.56	291.0	2.88	4.51
K^+	10.967	2.038	.179	14.759	7.568	5.40	63.7	2.01	6.47 (43.5)
K^+-free + db-cAMP	13.252	1.357	.170	15.058	6.262	10.00	76.1	2.59	4.51 (0.0)
K^+ + db-cAMP	11.911	1.658	.331	12.783	9.178	7.35	36.1	1.54	7.88 (74.7)

Addition of 4 mM K^+ brings about radical changes in these parameters. There is a decrease in the levels of ATP and PCr, along with increases in ADP, AMP and creatine. These changes result in the depression of the ATP/ADP, ATP/AMP and PCr/Cr ratios. Respiration also is greatly increased.

These modifications are similar to those observed under stimulation by secretagogues (Table IXa). Thus, the addition of K^+ to K^+-deprived cells, induces metabolical changes typical of secretagogue stimulation. These findings suggest that cells are in an active secreting state before being inhibited by K^+ withdrawal. Addition of K^+ may reinstate a previous secreting state. The mechanism is unknown by which these cells are stimulated without the exogenous addition of secretagogues. It is unlikely that endogenous secretagogues are present in an amount sufficient to induce stimulation. The possibility should not be discarded that the receptor may be activated by the proteolytic enzyme.

Addition of db-cAMP to K^+-deprived cells brings about an increase in ATP concentration, and a large decrease in ADP. In this case modification of the concentration of AMP does not parallel that of ADP; rather, there is an increase in the level of AMP. Alterations of adenine nucleotides are accompanied by relatively minor ones in the PCr/Cr couple: a small decrease in PCr and an increase in creatine is observable. Thus, changes in various ratios are not parallel. A large increase in ATP/ADP ratio occurs, whereas ATP/AMP and PCr/Cr ratios are depressed upon stimulation. Little or no response in respiration is elicited by adding db-cAMP to cells incubated in K^+-free solutions.

These findings indicate that in the absence of a functioning pump db-cAMP is able to stimulate the net synthesis of ATP, probably by increasing metabolism. Stimulation of glycolysis and lipolysis by cAMP is

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known to occur in a number of tissues. However, the increase in the ATP/ADP ratio (or metabolic rate) does not seem to be associated with oxidative metabolism, since in these conditions respiration is not increased by db-cAMP. Thus, substrate level phosphorylation is responsible for the increase in ATP and the decrease in ADP. The involvement of several different reactions has to be postulated to account for the results recorded for different metabolites. They may include ATP-forming reactions in the glycolytic pathway (phosphoglycerate kinase and pyruvate kinase), adenylate kinase reaction and the creatine phosphokinase reaction.

Again,adding potassium ion to the cells in the presence of db-cAMP (rows three to four) induces changes that are characteristic of the transition from the resting to the secreting state. There is a large decrease in ATP and phosphocreatine,in conjunction with increases in ADP, AMP and creatine. Therefore, a large drop in ATP/ADP, ATP/AMP and PCr/Cr ratios is observable. The modifications in high energy phosphate compounds are accompanied by a large increase in respiration. Hence, judged from the changes in the concentration of metabolites, the activation of the H^+ pump by adding potassium in the presence of db-cAMP leads to the increased utilization of high energy phosphate compounds.

Thus, both with and without the presence of db-cAMP, the addition of K^+ induces increases in respiration and effects in high energy phosphates that reflect activation of the H^+ pump. It is interesting to note that both the increase in respiration and the relative changes in high energy phosphate compounds are more marked in the presence of db-cAMP. This finding is not surprising since db-cAMP induces acid secretion in isolated intact mucosa (See Table VIII) and may exert larger effects in these parameters.

These results may indicate that at least in some preparations isolated cells are not in a truly resting state. However, the addition of db-cAMP is able to induce further stimulation. Furthermore, a pseudo resting state can be achieved by withdrawing K^+ from the suspension solution.

Comparison of the joint effects of K^+ and db-cAMP with K^+-free conditions should provide clues about the overall effect of stimulation (row four vs one). In this case, the effects are complex and probably reflect additive actions on both the H^+ pump and substrate mobilization. Thus, the addition of both K^+ and db-cAMP produces a decrease in ATP, ADP, and phosphocreatine, and increases the levels of AMP and creatine. Consequently, a large drop in ATP/AMP and PCr/Cr ratios occurs with a minor decrease in the ATP/ADP ratio. These changes are accompanied by a large increase in respiration.

The small decrease in the ATP/ADP ratio per se is not consistent with increased high energy phosphate utilization brought about by stimulation of the H^+ pump. However, the large drop in the ATP/AMP ratio indicates that the adenylate kinase reaction contributes to the overall energy balance.

The extent of this contribution is not known since steady state concentrations indicate little about turnover of these compounds. Thus, the ADP produced during stimulation can be converted again to ATP as follows:

$$2\ \text{ADP} \xleftrightarrow[\text{Kinase}]{\text{Adenylate}} \text{ATP} + \text{AMP} \qquad (13)$$

The ATP produced from this reaction would then be utilized by the H^+ pump.

Furthermore, in the creatine phosphokinase reaction, the large decrease in phosphocreatine may indicate a transfer of the phosphate group to ADP.

$$PCr + ADP \xleftrightarrow[\text{Kinase}]{\text{Creatine}} Cr + ATP \qquad (14)$$

ATP derived in this reaction is available for utilization by the H^+ pump. There is a net loss of nucleotides, indicating an increased turnover of these compounds induced by stimulation.

This experiment illustrates that db-cAMP acts on energy-producing, as well as energy-consuming reactions. Thus, the measured concentrations of metabolites may reflect changes in two different nucleotide pools; i.e., the cytoplasmic and mitochondrial.

The effect of cAMP on the H^+ pump and substrate mobilization may require different optimal concentrations that may explain why histamine has a more marked effect on the ATP/ADP ratio than db-cAMP does. If histamine increases cAMP concentration in the cell, it still is probably lower than that produced by the exogenous addition of 5 mM db-cAMP. Stimulation of energy-consuming reactions (H^+ transport) might then require a lower concentration of this compound.

Effects of oligomycin

The study of the effects on respiration by oligomycin reveals that this compound is able to block completely that fraction of oxygen uptake stimulated by db-cAMP. These experiments indicate that respiration coupled to phosphorilation is essential for stimulation, which suggests that normal ATP production may be required for the H^+ pumps to function.

Since in other tissues the inhibition of coupled respiration by oligomycin induces a fall in ATP and also inhibits some ATPases, including the K^+-stimulated ATPase of the bullfrog gastric mucosa (82), it was necessary to examine the effects of this compound on the levels of high energy phosphates in isolated oxyntic cells.

In the experiments shown above it was demonstrated that incubation with this compound for two and one-half hours completely inhibits the respiratory response to stimulation. Thus, the effect on the levels of high-energy phosphate compounds of two and one-half hr exposure to 10 µg/ml oligomycin was examined. Controls were studied at the same time (Table XI).

Addition of oligomycin causes a fall in ATP concentration to less than half of control values. This is accompanied by a large increase in ADP and AMP levels. Consequently, both the ATP/ADP and ATP/AMP ratios are largely depressed. Phosphocreatine levels fall to values almost 20 times lower than those of controls, which is matched by an equally large increase in creatine concentration. These changes effect a very large decrease in the PCr/Cr ratio. In this way they are similar to those induced by anoxia (40,47), which indicates that oligomycin is an effective inhibitor of phosphorilation (83,84).

Such a finding suggests that oligomycin inhibits acid secretion by blocking energy-producing rather than utilization processes. In this respect the effect produced by adding db-cAMP to cells inhibited by oligomycin has proved interesting: stimulation brings about a further decrease in ATP and phosphocreatine. Thus, the ATP/ADP and PCr/Cr ratios are reduced further. This compound does not prevent utilization of small amounts of the high energy phosphates still available. As has been reported for intact mucosa (45,75), it seems that a substantial fraction of ATP is not readily available to energy-consuming reactions. On the other hand, these levels may reflect a steady state maintained by glycolysis.

These observations confirm that oligomycin acts on mitochondria by blocking oxidative phosphorilation, and imply that the abolition of trans-

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TABLE XI. Effect of Oligomycin (10 ugr/ml) and db-cAMP (5mM) on adenine nucleotides in isolated oxyntic cells. Average of two experiments (cells pooled from 6 mucosae each).

	ATP	ADP	AMP	PCr	Cr	ATP/ADP	ATP/AMP	PCr/Cr
	(µMoles/gr d.w.)							
Control	12.673	1.303	0.026	17.395	06.311	9.73	487.0	2.76
db-cAMP	12.441	1.837	0.274	16.182	10.818	6.77	45.0	1.50
Oligomycin	5.564	4.482	1.003	.971	24.212	1.24	5.6	0.040
Oligomycin + db-cAMP	5.100	5.342	0.925	.405	27.818	0.95	5.5	0.015

port is dependent primarily on the inhibition of energy production, rather than utilization.

Investigation of the effects on the balance of energy phosphate compounds of stimulation by secretagogues and inhibition by oligomycin indicates that these compounds are utilized as energy sources in the normal secretory process. In isolated cells some of the effects may be masked by the multiplicity of actions of some secretagogues. However, the effects can be sorted out under the proper conditions.

E) Intracellular pH

Because Poole's et al. (52) original method prescribes extracting DMO into the organic phase from 5M NaH_2PO_4, it was necessary to check how efficient the extraction of DMO from 0.1 N HCl is. Figure 31a shows that the extraction of·DMO from 0.1N HCl is not complete. Thus, two extractions are used to extract 98% of the total radioactivity. Figure 31b illustrates that extraction from the cell pellet extract is less efficient than from the supernatant (87% vs 92%). However, when two extractions are performed, there is no significant difference between the two samples. Thus, the use of one sample for both inulin and DMO determinations has the advantage of requiring half the amount and it avoids one more potential source of error. It has been found that one of the biggest sources of error limiting the sensitivity of the method is the weighing of the wet and dry cellular pellets. By using one sample, only one weight (and its error) is taken into account for the calculation.

Extracellular pH is constant throughout the period of measurement of intracellular pH (60 min). In most of the experiments the value for intracellular pH is somewhat higher than the measured extracellular pH (Table XII). If all the results obtained during control conditions are

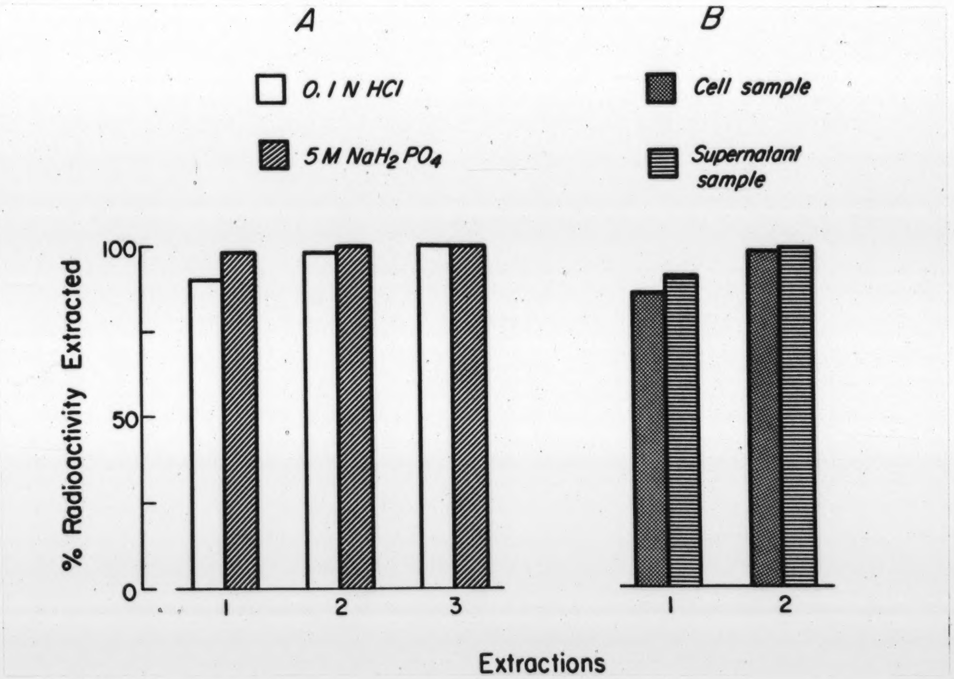

Figure 31: Check of the efficiency of extraction of DMO-^{14}C into the organic phase. A. Extraction from 0.1 NHCl as compared to 5M NaH_2PO_4 B. Extraction from cell pellet extract as compared to supernatant extract.

pooled, the mean value for intracellular pH is 7.187, which is distinctly higher than the value of 7.110 ($p < 0.025$) recorded for extracellular pH.

Figure 32 presents the results of five different experiments where the effects of stimulation with 5mM db-cAMP were studied. Figure 32a demonstrates that there is little change in the intracellular pH of control aliquots measured at 0, 20, and 60 min. The scatter in the absolute value of pH obtained in different experiments is due to small variations in extracellular pH from experiment to experiment. This variation probably occurs because of the complexity of the buffer that is used (20 mM TES + 18 mM HCO_3^-/5% CO_2 + 1% albumin), and because of the small and variable amounts of acid equivalent released into the medium by the isolated cells. Addition of 5 mM db-cAMP at 0 min increases intracellular pH in all experiments (Fig. 32b). This increase was already observed at 20 min and was maintained in the 60 min sample. Upon addition of db-cAMP in the experiment with open triangles, there is no increase in pH at 20 min, but there is an increase at 60 min. An error in the weighing of the cell pellet probably explains the dissimilar increases in pH.

Combined results of these five experiments are demonstrated in Table XIII. As was expected, intracellular pH at 0 min is the same in both control and stimulated conditions. Values obtained for control at 20 and 60 min do not differ from values at 0 min, which indicates the maintenance of a stable condition throughout the experimental period. Stimulation increases intracellular pH from 7.194 to 7.317 at 20 min, and it is kept at 7.311 for 60 min. These changes are statistically significant. Since there is considerable scatter in the different experiments, changes in

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TABLE XII. Relationship between intracellular and extracellular pH in isolated oxyntic cells in control conditions.

	(n)	pHi	"p"
pH-ext.(control)	(20)	7.110 ± 0.020	
pH-int.(control)	(20)	7.187 ± 0.023	<0.025

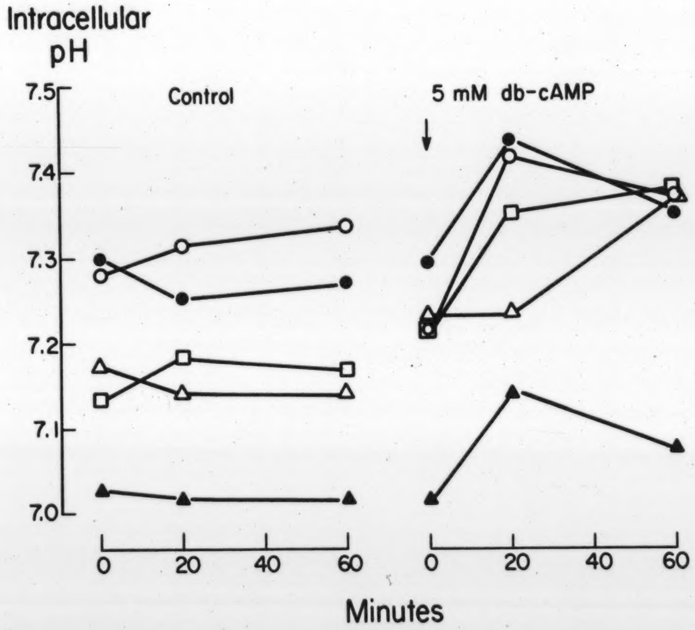

Figure 32: Intracellular pH in isolated oxyntic cells. Figure shows five different experiments. A. pHi measured in control aliquots at 0, 20 and 60 minutes. B. pHi measured at 0 minutes (control) and 20 and 60 minutes after the a-dition of 5mM db-cAMP.

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TABLE XIII. Effect of db-cAMP (5mM) on Intracellular pH in isolated oxyntic cells. n=5 Intracellular ph measured at 0 minutes and 20 and 60 minutes after addition of db-cAMP.

	pHi		p
	Control	db cAMP	(control vs stimulated)
0'	7.183 ± .050	7.194 ± .047	(n.s)
p(20'-0')	(n.s)	(<0.02)	
20'	7.182 ± .051	7.317 ± .056	(<0.005)
p (60'-0')	(n.s)	(<0.01)	
60'	7.187 ± .055	7.311 ± .059	(<0.05)

intracellular pH over a period of time are plotted in Figure 33. Apparently, the magnitude of the change does not depend on the initial value of pH: addition of db-cAMP induces a change of about 0.12 pH units. The error around each point in the controls is a measure of the sensitivity of the method, assuming the absolute stability of the pH under these conditions.

It is of **primary interest** to know if this change in pH is related to the stimulation of acid secretion in the isolated cells by db-cAMP and is not a product of some other action of the compound. In an attempt to answer the preceding question, the effects of pretreatment with 20 mM SCN^- on the pH response to db-cAMP, are studied (Fig. 34). Pretreatment with SCN^- annuls the increase brought about by db-cAMP, which indicates that acid secretion is necessary to induce a change in pH. Although this result must be interpreted with caution because it is derived from only one experiment, it should be pointed out that these values were obtained from the pooled cells of six bullfrog mucosae, that db-cAMP always increases intracellular pH (see Figure 13) and that, as was expected, the one pretreated with SCN^- resembles the behavior of the controls.

In summary, these results indicate that oxyntic cells alkalinize their intracellular space, as measured by DMO, when they are stimulated with db-cAMP. The change in pH seems to be dependent on the activity of the H^+ pump because it can be blocked by SCN. Thus, it is highly likely that the change in intracellular pH results from the active extrusion of H^+ from the oxyntic cells.

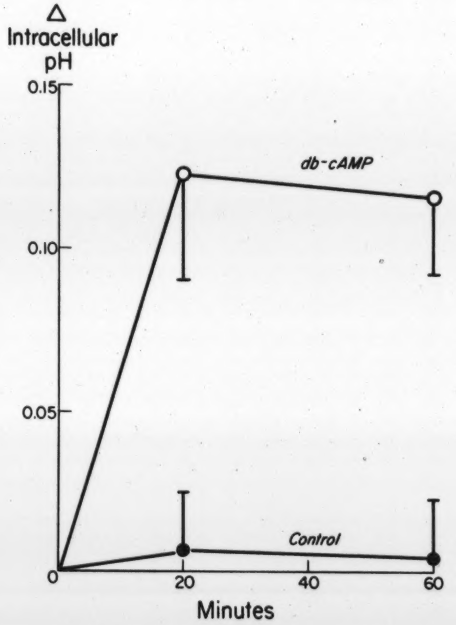

Figure 33: Change in intracellular pH as function of time in control conditions (-0-) and stimulated with 5mM db-cAMP (-0-). n=5

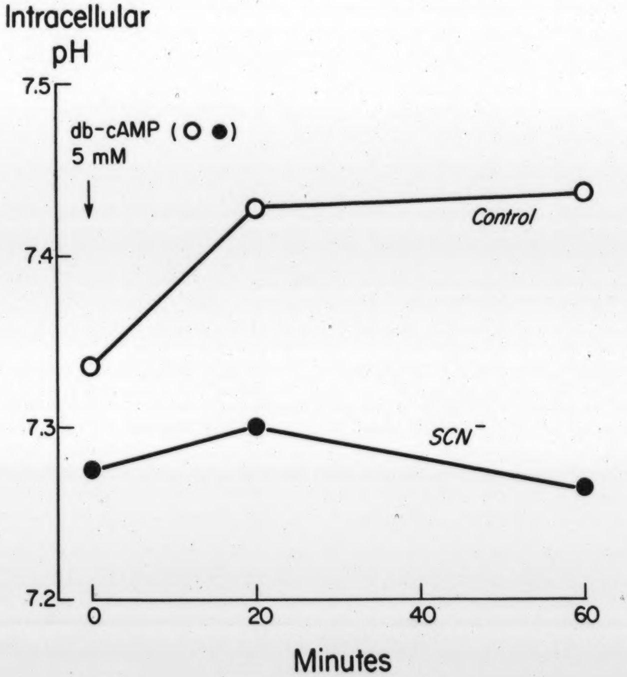

Figure 34: Effect of SCN$^-$ on the change in intracellular pH induced by db-cAMP. One aliquot of cells was incubated for 60 min with 20mM SCN$^-$. Intracellular pH measured at the end of this period (0 minutes) in control and SCN$^-$-treated aliquots. Db-cAMP was then added to both aliquots and intracellular pH measured 20 and 60 minutes after addition.

DISCUSSION

A) Cell Isolation and Purification

Enzymatic digestion of the mucosa by pronase combined with mechano-osmotic treatment renders a preparation containing more than 80% viable oxyntic cells. The procedures described previously combine essentially two different techniques. In order to improve the preparation's content of oxyntic cells, an additional purification step was added at the end.

Important modifications were introduced in the technique for necturus gastric mucosa originally described by Blum et al. (10). Mounting the mucosa in the lucite tube, instead of pinning it in a dish, was important for three reasons: 1) better oxygenation of the mucosa was provided throughout the isolation procedure (~ 3 hours); 2) the enzyme could be instilled only on the secretory or luminal side in this way providing for more controlled isolation; and 3) in this kind of set-up mechanical agitation (vortex) was made possible and it played an integral role in the isolation of the oxyntic cells.

In preliminary experiments it was found that enzymatic digestion of the whole mucosa was difficult to control. Furthermore, in the frog it did not produce the selective and sequential release of cells from the mucosa that Blum et al. reported for necturus: they had found that exposure of the mucosa to the enzyme resulted in the sequential and progressive detachment of cells. Epithelial cells detached first, then mucous neck cells and finally a preparation rich in oxyntic cells was obtained. In the case of the frog, the enzyme attacked the surface in a patchy pattern. Thus, areas of complete erosion were found along with intact ones. As a result, serial fractions of the cells appeared to contain an almost fixed proportion of oxyntic and nonoxyntic cells. On the other hand, enzymatic treatment caused epithelial cells to release their mucus, in turn inducing serious clumping problems. Histochemical techniques demonstrated that mucus was released from the epithelial cells. Staining isolated cells with P.A.S. and n-BT showed that most of the nonoxyntic cells stained very little with P.A.S., in distinct contrast with intact mucosa. Later this finding was confirmed by electron microscopic studies in which surface epithelial cells contained very few, if any, granules (compare Figures 17 and 9). This was not the case for mucous neck cells.

Elimination of epithelial cells was necessary before cells were exposed to the enzyme. For these purposes a technique described by Forte and collaborators (43) was used. Treatment with hyperosmotic solution selectively damaged epithelial cells so that they could be easily removed by gentle blotting and scraping. The mechanism by which hypertonic solutions disrupt mucus cells is unknown. Altamirano (91) has reported similar results in the mammalian stomach with an intact blood supply and he proposed that hypertonic solutions reversibly disrupt some permeability barrier in the membrane of mucus cells. Forte et al. (43) suggested that mainly the mucus granules and their limiting membrane were affected. Anyway, acid-secreting cells did not appear to be damaged by this procedure. In the present study, the described procedure did not induce changes in the oxyntic cells, and if it did, these modifications would appear to be reversible from the morphological and physiological point of view.

Thus, removal of the mucus cells provides a preparation that can then be treated with proteolytic enzymes in order to obtain oxyntic cells.

For the isolation of oxyntic cells, the amphibian stomach has ad-

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vantages over the mammalian mucosa. The natural stratification of the tissue allows for the employment of simple purification procedures because the body of the gland is formed almost exclusively by oxyntic cells. Parietal, chief and different kinds of endocrine cells are found in the mammalian gastric gland.

In addition to the removal of mucous cells by the mechano-osmotic treatment, the cell preparation undergoes other processes for final purification. Low speed centrifugation of the cell suspension collected in the enzyme solution eliminates most of the debris and broken cells. The remainder is eliminated during the process of velocity sedimentation. Amsterdam and Jamieson (13) have reported similar observations for the isolation of pancreatic acinar cells. In this procedure, the relative sizes and density characteristics of oxyntic cells are utilized advantageously: they sediment at a faster rate because they are larger and denser in relation to the other types of cells present. Increasing the density of the medium by using 10% F.C.S. and 2% albumin increases the resolution of the procedure. This probably occurs because P-Po becomes much smaller for the nonoxyntic cell types (see Equation 11). Ideally, the density of the medium could be adjusted to the buoyant density of the contaminant cells; then P-Po would be zero and the cells would float. It is possible that the use of a 1 g sedimentation procedure in a velocity sedimentation apparatus (105) would provide a means by which different types of cell could be separated distinctly. This may be especially pertinent if studies need to be performed in other types of cell in the preparation that are less numerous.

Density gradient sedimentation has been used in the mammalian stomach to obtain preparations with a large proportion (65%-95%) of acid-secreting cells (4,5). The procedure was not attempted in the present study because of the serious clumping problems associated with this type of purification. Procedures that are relatively simpler yielded comparable results without the disadvantages mentioned above.

The cell isolation procedure obtained a high yield of oxyntic cells. About 60% of the oxyntic cells initially present in the tissue can be recovered with a high viability ($>$ 90%). This indicates that only a small amount of the oxyntic cells may be actually disrupted in the isolation, the remainder is probably lost during washing and purification.

B) Oxygen consumption studies

The rate of oxygen uptake by isolated cells has been employed as an index of functional viability in a number of tissues. Moreover, in some instances it has been possible to correlate oxygen uptake with a particular physiological characteristic of a given cell type. In the present study the rate of oxygen uptake has provided useful information regarding the metabolic and physiological properties of this preparation.

For up to four hr, oxygen uptake by isolated cells is rather stable, the longest time followed. In control conditions this rate is about 4.6-4.8 μl/mg dw hr, which is much higher than that recorded for intact mucosa (0.5-3.1 μl/mg dw hr) under different conditions (25). The basal rate of oxygen uptake in this study is similar to that reported by Blum et al. (10) for necturus isolated oxyntic cells. It is much lower than that reported for mammalian parietal cells (7), which is about 17 μl/mg dw hr, but it is higher than in other amphibian tissues (92). This is not surprising since mammalian cells contain a larger proportion of mitochondria (54) and respiration is assayed at 37°C instead of 25°C. Also, oxyntic cells probably have a faster rate of oxygen consumption than toad bladder (92) for example, because of the greater number of mitochondria. The same interpretation applies when the rate is compared to that for intact mucosa. Another explanation would be that the isolated cells are damaged and become uncoupled. Experiments with metabolic inhibitors, such as DNP, specifically, are illuminating in this regard.

The effect of metabolic inhibitors (Table 1) provides some insight into the general metabolic characteristics of the preparation. Amytal (2 mM) inhibited respiration by 70%. Similar results have been obtained in intact mucosa (93), which suggests that some of the respiration is linked to oxidation of succinate. Nevertheless, this respiration in intact tissue could not support the active transport of H^+. The effects of azide (5 mM) were unexpected since there was an increase, in respiration, rather than a decrease.

Often, azide can act as a blocker of electron transport or as an uncoupler (85). The increase in respiration brought about by azide indicates that this compound acts as an uncoupler in isolated cells. At similar concentrations azide can inhibit active transport and respiration in intact mucosa (67). This discrepancy might exist because isolated cells are more permeable and the presence of higher intracellular concentrations may uncouple rather than inhibit electron transport. On the other hand, a species difference in the response could account for the results; Sachs and collaborators used Rana pipiens; whereas Rana catesbeiana was used in the present study.

DNP increased respiration more than threefold, which is much greater than the effect obtained in intact mucosa (23, 65), in turn indicating that isolated cells can be uncoupled.

The way in which secretagogues affect respiration has provided some

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information about the mechanism of stimulation of the secretory process. Pentagastrin (PG) does not stimulate respiration in the isolated cell preparation. This is consistent with the hypothesis that PG acts through an intermediate, such as histamine. Kasbekar's (95,96) and Rangachari's (59) work supports this theory. Kasbekar has shown that mucosae can be rendered tachyphyllactic to Pentagastrin by repeated stimulation. If the mucosae are then incubated with subthreshold amounts of histamine, the response to PG can be recovered. Presumably histamine was exhausted from a pool that could be replenished by incubation with low doses of this compound. Then this pool would be available again for release by PG. In fact, Rangachari (59) measured a release of histamine by PG and also by acetylcholine. Thus, histamine liberated by PG from stores outside the oxyntic cell would be diluted into the large suspension volume. As a result, the compound would not reach threshold levels. Alternatively, selective inactivation of a PG receptor on the oxyntic cell could account for the lack of response to PG.

Addition of histamine results in a small but significant increase in oxygen uptake that is less than the relative increase in respiration elicited in the intact mucosa. There may be several explanations for this result. Firstly, the "high" response in intact tissue may be accounted for by histamine stimulating other cell types in the intact mucosa. Secondly, the histamine receptor may have been damaged by the isolation procedure. Nevertheless, experiments showed that exposure to pronase for up to one hr did not prevent a sizable response to histamine. On the other hand, the response was not improved by incubation in a supplemented media in order to regenerate the receptor. Thirdly, histamine may be acting on top of a high baseline respiration. In other words, the cells may somewhat be in a secreting state before the addition of histamine (vide infra, Adenine nucleotide experiments). If this is the case, it is probably not caused by the extant histamine. Repeat washes and exposure to burimamide, an antagonist of the H_2-receptor, do not change basal respiration. This compound completely blocks the stimulation of acid secretion brought about by supramaximal doses of histamine in the intact mucosa (62). However, pronase possibly irreversibly activates some receptors, in this way inducing a state of basal secretion that is not sensitive to an H_2-receptor antagonist. It is interesting to note that pronase stimulates acid secretion in the intact mucosa. However, it is not known whether stimulation was the direct result of an effect on the oxyntic cell or was mediated through the release of a secretagogue, such as histamine.

The results with acetylcholine were somewhat unexpected. If this compound acts solely by releasing histamine, the degree of stimulation of respiration should be similar to histamine. Instead, it is larger. Furthermore, the effects of histamine and acetylcholine on respiration appeared to be additive, implying that acetylcholine acts on the oxyntic cell, in addition to releasing histamine. The mechanism of action by which acetylcholine directly stimulates the oxyntic cell is not known. Elevation of cGMP levels in response to cholinergic stimulants has been reported (88).

At this point, it should be noted that Nakajima et al. (89) and Forte et al. (97) have found that adenyl cyclase purified from amphibian gastric mucosae was stimulated by gastrin. On the other hand, Rangachari (59) has found that histamine is liberated by both acetylcholine and pentagastrin in sufficient amounts to account for the stimulation of acid secretion.

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Thus, in addition to inducing the release of histamine , secretagogues such as PG and Ach may directly activate a receptor in the oxyntic cell, and in this manner modulate the response.

If secretagogues increase intracellular levels of cAMP, receptors can be bypassed by the direct addition of this compound. In this study db-cAMP (5mM) elicited the largest increase in respiration (40%) of all the secretagogues used. Stimulation of respiration is similar, if not larger, than that observed in intact mucosa (26). Thus, even if some receptors are damaged by isolation, the large increase in respiration elicited by db-cAMP indicates that the secretory apparatus is intact.

Experiments with inhibitors of acid secretion (Tables III-V) suggest that more than half of secretagogue-induced respiration is abolished under conditions where acid secretion by isolated cells is expected to be negligible. Thus, SCN^- inhibits respiratory response to acetylcholine and db-cAMP by more than 50%. Similar results have been obtained when Cl^--free solutions are used to block db-cAMP-induced respiration.

These results indicate that at least a large fraction of the respiration increase induced by secretagogues is associated with acid secretion by isolated cells, which essentially is similar to Harris' and Alonso's (25,26) findings in mucosal minces from Rana pipiens. The inhibitor-insensitive respiration could be caused by a number of processes. For example, residual Cl^- in the solutions may explain why some acid is secreted by the cells. However, if we take into account the kinetic dependence of acid secretion on Cl^- concentration (98), less than 10% of the secretion remains at the Cl^- values that are found in the solutions (1mM). On the other hand, this does not explain effects of SCN^- that presumably are "specific". The mechanism of action of SCN^- is not known and at the same time may affect the pump and metabolism (27). Nevertheless, it should be noted that SCN^-- and Cl^--free compounds inhibit the respiratory response to Ach and db-cAMP to the same extent. Thus, the effects of SCN^- on the pump may be more important than the effects on metabolism, at least as far as this experiment is concerned. Stimulation of metabolic processes by cAMP seems a plausible explanation for inhibitor-insensitive respiration. This compound can stimulate glycolysis and lipolysis in many tissues including the gastric mucosa. Harris and collaborators (63,64) have found that cAMP stimulated glycogenolysis in intact mucosa. In addition High and Hersey (65) found that theophylline reduces the respiratory quotient in gastric mucosa, which indicates that cAMP mainly increases the oxidation of lipid substrates.

Withdrawal of K^+ from the bathing solutions completely abolishes respiratory response to db-cAMP. This form of inhibition blocks the fraction of respiration associated with activation of the H^+ pump, as well as the fraction of respiration attributed to substrate mobilization. Thus, K^+ may be essential not only for the pump, but also for mitochondrial oxidation.

In this respect, some preliminary experiments with substrates have proved interesting. Alonso and Harris (25,99) have shown that the gastric mucosa can oxidize fatty acids at the same rate in Cl^-, as well as in Cl^--free solutions, the present study of isolated oxyntic cells confirms the preceding results. On the other hand, K^+ withdrawal inhibits by more than 50% oxidation of β-oH-Butyrate by isolated oxyntic cells, which indicates that this ion is necessary for the normal oxidation of substrates. Thus, under these conditions both the pump and mitochondrial oxidation are inhibited and db-cAMP cannot effect an increase in oxygen uptake. The mechanism by which K$^+$ withdrawal inhibits oxidative metabolism in isolated cells is not known. Nevertheless, possibilities will be discussed in relation to the experiments on adenine nucleotides.

The study of the effects of oligomycin on respiration provides information concerning the mechanism of energy coupling to acid secretion. The increase in oxygen consumption brought about by db-cAMP is completely inhibited by oligomycin, a well-established inhibitor of oxidative phosphorylation. This compound selectively blocks the fraction of respiration that is coupled to phosphorylation. However, it does not have any effect on uncoupled respiration, as was shown by the addition of DNP, which elicits a large increase in respiration in the control, as well as in the oligomycin-treated chamber. Basal respiration is affected only slightly indicating that a large fraction of oxygen consumption in resting conditions is not coupled to oxidative phosphorylation. Inhibition of db-cAMP-stimulated respiration is dependent on time: exposure to oligomycin for 1.5 hr reduces, but does not abolish, the response, whereas the increase in respiration is blocked completely when oligomycin is present for 2.5 hr. This may indicate low permeability to the compound. These effects of oligomycin on respiration are accompanied by the expected changes in adenine nucleotides. Thus, the reduction in ATP and PCr, and the increase in ADP, AMP, and Creatine reflects the inhibition of phosphorylation. A very interesting finding is that db-cAMP further reduces the level of ATP and PCr. This can be interpreted as an increase in the utilization of ATP by stimulation of the H$^+$ pump. Furthermore, this indicates that the pump is not inhibited "per se" and can function in spite of very low levels of ATP. A substantial fraction of ATP is not affected by oligomycin or stimulation in oligomycin-inhibited cells, which concurs with earlier reports (45,75,76).

These compounds would not be readily available to energy consuming reactions. On the other hand, they may reflect a steady state maintained by glycolysis in either oxyntic or contaminant cells or both. Thus, the inhibition of acid secretion in the intact mucosa and secretagogue-stimulated respiration in oxyntic cells by oligomycin are related to inhibition of the production of ATP and do not seem to be related to a secondary effect of this compound.

In summary, experiments using oligomycin illustrate the following.

1) a large fraction of basal respiration is not coupled to phosphorylation. It is possible that the energy dissipated in substrate oxydation in basal conditions is utilized by other processes such as mitochondrial ion transport.
2) Permeability to oligomycin may be limiting: thus, long periods of exposure are required to achieve inhibition.
3) Db-cAMP-stimulated respiration is coupled entirely to phosphorylation.
4) As was expected, oligomycin reduces ATP and PCr because of its properties as a phosphorylative inhibitor. This effect, however, does not preclude utilization of small amounts of ATP and PCr still available to the H^+ pump.
5) Oligomycin does not block uncoupled respiration as was indicated by the large increase in oxygen uptake induced by DNP.

These observations support the theory that ATP is the immediate energy donor for the secretory process.

C) Ultrastructural studies

In intact mucosa studies were performed for two reasons: 1) the correlation and comparison of the morphology of cells before and after

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isolation; and 2) the investigation of the effect of stimulation with db-cAMP on the morphology of oxyntic cells.

The surface of the stomach is lined with surface epithelial cells that invaginate into the pits. Two or three glands consisting almost entirely of oxyntic cells empty into a pit. The gland-pit transition, the so-called neck of the gland, is lined with mucous neck cells. Argentaffin cells are found around the mucous neck cells and scattered along the body of the gland.

The results reported for the morphology of the gastric mucosae in the resting state essentially are similar to those reported earlier by various authors (see ref 30).

Oxyntic cells border the lumen of the gland. The apical surface is smooth, but has a few microvilli. A typical tight junction (73) holds together oxyntic cells at the apical border. No desmosomes or gap junctions are seen in the intercellular space, thus permitting the intercellular spaces to change dimension. It has been proposed that this space plays a role in the water flow accompanying H^+ extrusion (94). The apical pole of resting oxyntic cells is packed with vesico-tubular membranes. As has been reported by other authors, these membraneous structures do not come into contact with the apical plasmalemma. The nucleus and the mitochondria are displaced toward the basal pole of the cells. Lipid droplets are abundant in the oxyntic cells and may represent the stores from which fatty acids are used in the normal secretory process (98).

Stimulation of the intact mucosa with 5 mM db-cAMP brings about radical changes in the structure of oxyntic cells that are similar to those where histamine is used as a stimulant (29,30). Also, analogous alterations have been found in mammalian parietal cells when the mucosa is stimulated

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by a variety of agents(99-102)The apical plasma membrane develops numerous microvilli and foldings, thus increasing the plasma membrane area because of the fusion of tubulo-vesicles with the apical plasmalemma (29,30)

This process, however, seems to involve a dynamic turnover. In other words, membrane fusion and retrieval take place at the same time (37,38) with a net increase in steady-state surface area.

Thus, db-cAMP induces membrane transformations found in other types of stimulation. Since other types of stimulus may induce at the end a rise in cAMP levels, this finding implies that membrane proliferation may be essential for the secretory process to occur. Actually, in the mammalian mucosa the increase in plasma membrane area appears to precede the onset of secretion (103). The significance of this finding is not known.

Separation from the tissue causes some alterations in the cell structure.

The surface epithelial cell does not appear to lose its polarization in the sense that secretory granules are localized in one pole of the cell and the nucleus in the other. Similar observations have been reported for pancreatic acinar cells, which also release granule content by exocytosis (14). Granules, however, appear to be less packed than in the surface cell of the intact mucosa. Also they appear to be situated farther from the surface of the cell. The decrease in the amount of granules and the packing may reflect granule release during the isolation procedure, and it is not known at what rate they can be resynthesized. Their distance from the surface of the cell may signify that there is an absence of stimuli for normal discharge. Numerous hemidesmosomes in the walls of phagocytic vacuoles indicate that membrane specializations undergo degradation and metabolism inside the cells when these structures are no longer functional. Perhaps other membrane components undergo the same catabolic process.

All mucous neck cells examined showed an impressive degree of polarization (compare Figs. 10 and 18). In this case an apical pole is recognizable because of the presence of numerous microvilli. The apical pole of the cell is packed with a large number of granules. Clearly this cell contains many more granules than its counterpart in the intact mucosa. Also, as has been observed for the surface epithelial cells, the granules seem to be farther from the surface. These two observations suggest that in isolation these cells do not release their granules, perhaps because of the lack of normal stimuli for granule discharge. In the few cells observed, addition of db-cAMP does not seem to alter the morphology of mucous neck cells or epithelial cells. It should be pointed out that acetylcholine and vagal stimulation induce mucus release (104). Also, nerve terminals are abundant in the vicinity of mucous cells (see Fig. 18).

The few argentaffin cells observed in these preparations did not appear to have undergone any change in the isolation procedure. In this sense it is much more difficult to recognize polarization.

Studies on isolated oxyntic cells in the resting state showed that these cells are able to maintain a large degree of polarization even a few days after separation from the intact tissue. Thus, it has been observed that the relationship between the different cell organelles very closely resembles that present in the oxyntic cell in the framework of the tissue. The tubulo-vesicular membranes occupy one pole of the cell while the nucleus and mitochondria are at the other pole. Furthermore, the proportion of tubulo-vesicles appears similar to that in the intact mucosa. The surface membrane seems fairly smooth with a few microvillous projections. In some cells these projections resemble foldings seen in the intercellular space. The findings indicate that the topography of the cell is maintained after isolation, which suggests that the spatial organization in the tissue is not essential for the maintenance of well-defined structures.

However, it is not yet known whether the different regions of the plasma membrane will undergo randomization. In other words, will the integral proteins in the apical membrane diffuse laterally and intermix with the components in the basolateral membrane, and vice versa? According to the fluid mosaic membrane structure (105), proteins would be free to diffuse along the plane of the membrane in the liquid lipid matrix. This has been shown to happen in a number of biological and artificial systems (107). However, it is not known whether the diffusion of certain proteins is limited by "anchoring" with other submembranous proteins. This is so for erythrocyte proteins "anchored" by spectrin (108) and may be the case for integral proteins in other systems (S. J. Singer, personal communication).

On the other hand, the lipid composition of the apical membrane may be very different from that of the basolateral membrane so that little intermixing of the two lipid phases may occur. Because of very specific protein-lipid interactions, the integral proteins would not be allowed to cross from one region of the membrane to the other, and, in this way, the differential topography of the cell envelope would be maintained. Another possibility is that the membrane modification at the zonula ocludens remains in the membrane (hemidesmosomes are phagocytized) establishing a physical barrier that precludes randomization of components of the membrane. These questions are still to be investigated.

Addition of db-cAMP induces changes in the structure of the oxyntic cells that qualitatively resemble those occurring in the intact mucosa upon stimulation of an acid secretion. Essentially there is a striking proliferation of plasma membrane appearing in the form of long and slender foldings and microvilli and accompanied by an almost complete disappearance of the tubulo-vesicular membrane components. Thus, it would seem that in most oxyntic cells there was a complete fusion of these components with the plasma membrane. This raises the question as to whether the proliferation of membrane in the intact mucosa is physically limited by the size of the lumen. In this case it is much more difficult to recognize definite polarization of the oxyntic cell since the intracellular membranes are absent and because proliferation seems to take place on most of the surface of the cells making it difficult to ascertain which side corresponds to the apical pole. Since the plasma membrane is not a rigid structure, it is possible that new membrane formed at the apical side of the cell spreads around the entire surface of the cell creating more and more sites where vesicle-membrane fusion can occur. This would explain why the fusion of vesico-tubules is almost complete in the isolated cell. The presence of tight junctions would bar the spread of new plasma membrane in the intact tissue. It is not known whether vesico-tubules will fuse with the basolateral membrane in isolated cells. However, this probably does not occur since the membrane fusion reaction requires certain characteristics of membrane charge and lipid composition (109) that may not be met by the two different membranes. Moreover, electron micrographs always show areas where proliferation does not occur, further suggesting that this may be the case.

Thus, stimulation with db-cAMP produces morphological changes in the oxyntic cells that in principle resembles those modifications occurring in the intact tissue upon stimulation of acid secretion. Although these findings do not prove that isolated cells secrete acid, they strongly suggest it. In intact mucosa acid secretion is always accompanied by the same changes. On the other hand, the alterations do not occur, or are reversed, when acid secretion is inhibited by anoxia (110), K^+ withdrawal (78), or by permitting the mucosa to return to the resting state (30).

By using stereological techniques (111), Helander et al. (54) have quantitated the changes in surface area that occur upon stimulation. However, in isolated cells application of these techniques is difficult for the reasons already discussed in Results. Thus, the use of ectoenzymes as membrane markers provides a tool for the semiquantitation of alterations in membrane surface area. Essentially, the findings of Durbin and Kircher (51) were confirmed by this study. They found a correlation between enzyme activity on the luminal side and the expected changes in surface area, suggesting that the enzyme was not affected directly by the agents used to modify the secretory activity, but rather by the increase or decrease in the number of catalytic sites available at the surface of the cell. One can envision this process occurring at the expense of membrane fusion and retrieval. In isolated cells three different agents known to increase surface area in the intact tissue, and isolated cells (at least for db-cAMP),induce changes in phosphatase activity. Furthermore, these changes correlate highly with oxygen uptake by the isolated cells. This finding suggests that the magnitude of the secretory response corresponds to changes in surface area and metabolism. In other words, the rate of acid secretion depends on how much membrane can be exposed to the exterior at any given time or vice versa. On the other hand, the increase in metabolism (oxygen uptake) is secondary and dependent on these processes.

Thus, stimulation of isolated cells induces parallel changes in structure and metabolism that suggest that the isolated cells are able to secrete acid. The magnitude of the alterations in surface area may provide a means for controlling the number of sites available for H^+ translocation and in this way control the rate of acid secretion.

D) Adenine Nucleotides

Studies on adenine nucleotides in the intact mucosa show that db-cAMP induces changes similar to those observed under stimulation with histamine and theophylline. There was an increase in ADP levels causing a decrease in the ATP/ADP ratio; phosphocreatine levels rise bringing about a concomitant increase in the PCr/Cr ratio; and AMP levels increase producing a decrease in the ATP/AMP ratio. These results indicate that some of the ADP is reconverted to ATP through the adenylate kinase reaction: thus, the magnitude of the decline in the ATP/ADP ratio as compared to that occurring in the intact tissue is concealed by the contribution of the adenylate kinase reaction. Stimulation of acid secretion by db-cAMP augments the utilization of ATP, in turn producing more ADP and AMP. The rise in ADP levels may increase phosphorylation in the oxyntic cell mitochondria (112), thereby providing a mechanism by which secretagogues stimulate oxygen consumption (25). Secretagogues (cAMP in this case) increase the activity of the pump, in this way, enhancing ATP utilization.

In isolated mitochondria the addition of ADP leads to oxidation of certain respiratory chain members provided the substrate is not limiting

(113). However, in the gastric mucosa, stimulation with histamine produces a reduction in all respiratory chain members (114). This result has been interpreted as evidence against the involvement of ATP in acid secretion since Hersey (115) has claimed that an ATPase mechanism would predict oxidation of the respiratory chain. Substrates, however, may be limiting and, in addition to stimulating the pump, secretagogues may make substrates more available to the respiratory chain. Studies of respiration and those with K^+-free solutions in isolated cells, which will be discussed later, indicate that this may be the case.

Phosphorylation of creatine accompanies the changes in adenine nucleotides. At first this does not seem compatible with the increase in high energy phosphate utilization in response to stimulation. Durbin et al. (40), however, suggested that changes in the acidity of the cytoplasm and/or compartmentalization of some reactions may account for the contradictory behavior of the ATP/ADP and PCr/Cr couples.

Measurement of high energy phosphate compounds indicates that isolated cells can maintain normal concentrations of these compounds. Furthermore, ATP/ADP and PCr/Cr ratios have values similar to those found in the intact mucosa indicating that isolated cells metabolize normally.

The effects of stimulation on the levels of high energy phosphates presents a complex picture. Initial experiments (Table IX) show that in some preparations db-cAMP induces changes in part similar to those in intact mucosa. Thus, a decrease in ATP and an increase in ADP and AMP has been found, in turn producing a decline in both the ATP/ADP and the ATP/AMP ratios. These findings are essentially similar to those for the intact mucosa, except that ATP decreases with stimulation. A small but consistent decrease in ATP may have been masked in intact mucosa by

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the variability between the two halves. Alternatively, an opposite effect in another cell type in the intact mucosa might cause this small discrepancy.

In isolated cells the phosphocreatine-creatine couple changes in the direction contrary to that in intact tissue. Thus, PCr decreases and creatine increases causing a decline in the PCr/Cr ratio: a result more consistent with an overall increase in high energy phosphate utilization induced by cAMP. The discrepancy between intact mucosa and isolated cells perhaps exists because stimulation induces a larger energy requirement in isolated cells than in intact mucosa. This seems to be the case from the electron-microscopy studies, if we assume that modifications in the structure reflect the secretory activity. This would appear to be true from experiments with ectoenzymes where surface area, as measured by ectoenzyme activity, correlates highly with oxygen consumption. Alternatively, it is possible that alterations in a cell type other than the oxyntic cell dominate those measured in the intact mucosa.

Thus, these results indicate that stimulation of isolated oxyntic cells induces changes in adenine nucleotides similar to those produced by the stimulation of acid secretion in intact tissue. In addition, modifications in PCr and creatine are consistent with an overall increase in energy utilization. The movement of these alterations indicates that cAMP augments the activity of the pump, thereby increasing consumption of high energy phosphate compounds, but not precluding other effects of cAMP on energy-yielding reactions, which will be considered later.

In some experiments (Table IXb) no major change in nucleotide levels or ratios has been found upon stimulation. Possibly these negative results (cf. Results) indicate that cells were in a secreting state before being stimulated. On the other hand, cAMP may induce changes in energy-yielding as well as energy-consuming reactions, by which the effects on different metabolites may cancel out.

Experiments in K^+-free solutions and the effects of K^+ readdition and stimulation by db-cAMP may explain some of the preceding findings, as well as the overall effect of secretagogues. Withdrawal of K^+ inhibits acid secretion in the intact mucosa (77). In this case the morphology of the oxyntic cell resembles that observed in resting conditions (78). In addition, K^+ withdrawal reverses the changes on the ATP/ADP ratio induced by stimulation in the intact mucosa (40).

K^+-depleted cells maintain normal concentrations of high energy phosphate compounds, which indicates that K^+ is not essential for energy-yielding reactions. However, it is possible that a low rate of phosphorylation maintains such levels of high energy phosphates in conditions where energy utilization is rather low. However, a certain rate of phosphorylation is necessary for the maintenance of normal nucleotide levels since glycolysis alone is not able to do so (40).

Addition of K^+ induces changes on high energy phosphate compounds similar to those produced by db-cAMP stimulation and may indicate that in these experiments cells are in a secreting state and that K^+ withdrawal inhibits stimulation, reversing the effects on metabolites. This is also suggested by the large increase in respiration elicited by again adding K^+ to K^+-depleted cells. It is not known why cells may have been irreversibly stimulated without the exogenous addition of a secretagogue. However, addition of pronase induces acid secretion in the intact mucosa and, in this case, may have modified a receptor on the oxyntic cell. However, no one knows why this happens selectively in some preparations.

Anyway, if a pseudo-resting state is achieved by K^+ withdrawal, stimulation (by adding K^+) brings about changes that indicate activation of the H^+ pump.

Addition of db-cAMP to K^+-depleted cells induces changes in high energy phosphate compounds that reflect certain energy transformations. ATP increases and ADP decreases, augmenting the ATP/ADP ratio. This indicates that in the absence of a functioning pump db-cAMP promotes the net synthesis of ATP. Also, AMP increases and modifications in the phosphocreatine/creatine couple produce a small decline in the PCr/Cr ratio. Nevertheless, these changes in nucleotide concentrations are not accompanied by any alteration whatsoever in oxygen uptake. This suggests that ATP synthesis is not associated with oxidative phosphorylation, but with substrate level phosphorylation. ATP synthesis can be accounted for by ADP phosphorylation at the level of the adenylate kinase reaction (there is an increase in AMP) and creatine kinase reaction (there is a decrease in PCr and an increase in Cr) (cf. Equations 13 and 14).

The lack of response in respiration to db-cAMP in the absence of K^+ contrasts directly with the effects of this compound on oxygen uptake in conditions where the H^+ pump has been inhibited by other means, such as Cl^--free solutions and SCN^-. In these instances stimulation produces a sizeable response in respiration that presumably results from substrate mobilization induced by cAMP. Thus, in addition to its role in the activation of the H^+ pump, K^+ may be necessary for mitochondrial oxidation and other metabolic processes. Intracellular K^+ concentrations have been considered important in the regulation of the rate of glycolysis in ascites tumor cells (116-117). In isolated systems, large number of enzymes, including several glycolytic enzymes, are sensitive to K^+ concen-

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tration (118). The role of K^+ in mitochondrial metabolism has been difficult to assess. In isolated mitochondria K^+ affects the permeability to substrates (119-120), respiratory control (121), the P:O ratio (122) and adenine nucleotide translocase (123). In intact cells K^+ deficiency affects respiration (124) and reduces ATP levels (125), probably by diminishing the availability of substrates for mitochondrial oxidation. The mechanism by which K^+ deficiency may inhibit metabolism in isolated oxyntic cells has not been ascertained. It is interesting to note, however, that K^+ depletion inhibits respiration to a small extent (as compared to control values Table II). In addition, adenine nucleotide levels are normal in K^+-depleted cells both in intact mucosa (40) and isolated cells. In preliminary experiments, however, (not reported in Results) K^+ depletion inhibited the respiration induced by β-OH-butyrate. Nevertheless, the small response to β-OH-butyrate in K^+-free conditions was associated with an increase in ATP. Since basal respiration largely is not inhibited by K^+ depletion, and db-cAMP-stimulated respiration is totally blocked by the absence of K^+, this ion possibly may be needed for the oxidation of a particular substrate mobilized by cAMP, presumably fatty acids. It is interesting that oxidation of β-OH-butyrate is inhibited by K^+ deficiency. The site of potassium sensitivity may be situated at the level of β-oxidation or in the citric acid cycle (124). Normal levels of high energy phosphate compounds may be maintained by oxidation of substrates derived from other sources, or by a slow flow of reducing equivalents through the respiratory chain in conditions where the pump is inhibited and the total energy demand is expected to be very low. It is clear that more work is necessary to elucidate the role of potassium in the metabolism of the oxyntic cell. Since it has been argued that the primary action of secretagogues in stimulating acid secretion is on metabolism (27), it is necessary to investigate more thoroughly the role of K^+ in the direct activation of the H^+ pump, as has been proposed by Ganser and Forte (79,80) and by Scholes (81). However, changes in high energy phosphate compounds upon stimulation are not consistent with substrate mobilization as being the sole and primary action of secretagogues; rather, they indicate activation of the H^+ secretory mechanism.

The rate of oxygen uptake on the addition of K^+ is much larger than that in control conditions in other series of experiments (see Table II). Presumably, these two conditions should be equivalent. The rapid rate of respiration may indicate that in this set of experiments cells are stimulated before K^+ withdrawal and that again adding K^+ restores the previous secreting state. An alternative explanation is that respiration is not measured in steady-state, but, during a transient overshoot, due to the accumulation of certain intermediates during the K^+-depletion period.

In summary, addition of K^+ (with or without db-cAMP present) induces changes reflecting activation of the H^+ pump. The results are consistent with an overall increase in energy utilization induced by stimulation. At the same time, cAMP promotes a rise in energy production that may mask some of the effects of increased energy utilization. These findings indicate that secretagogues induce primary utilization of ATP by the pump.

E) Intracellular pH

The activity of H^+ in the cytoplasmic compartment depends on numerous variables: such as, distribution of H^+ across the membranes of the cell and different organelles, and the buffering capacity of the cytoplasm. The value of 7.194 obtained for overall cell pH in this study confirms earlier results in isolated parietal cells (7). This value is relatively high in comparison with the intracellular pH of skeletal muscle (126). However, the relative alkalinity in the oxyntic cell may be related to this cell's ability to secrete H^+. It is interesting that toad urinary bladder has a relatively high pH when measured by the same technique (127), and it is known that this epithelium secretes H^+ (128).

The constancy of intracellular pH in control conditions throughout the experimental period indicates that cells are in a steady state.

The active extrusion of H^+ to the extracellular spaces implies that hydroxyl ions remain inside the cell. Presumably OH^- is neutralized by the CO_2-HCO_3^- system, with the eventual release of HCO_3^- at the serosal side. In the steady state, H^+ secretion is accompanied by a stoichiometric release of HCO_3^- (18). However, a shift in intracellular pH is expected since alkaline release is a passive mechanism. In the present study a pH shift of about 0.15 pH units has been measured upon stimulation with db-cAMP. Such a change implies that isolated cells secrete acid in response to stimulation. Furthermore, the finding that pretreatment with SCN^- abolishes the change in intracellular pH induced by stimulation indicates that proton translocation across the cell membrane is necessary for pH to change.

Recently, Villegas et al. (129) have reported a change in intracellular pH in intact mucosa in response to stimulation. They attest that the apparent change in pH is due to an exteriorization of the vesicotubules that are filled with a fluid of low pH. Although such phenomenon may occur, this implies that the change in pH is not real, but an artifact caused by the reduction in relative cell volume. If the change in cell volume occurs as a consequence of stimulation, it is not large enough to be measured. Wet/dry ratios appear to be the same in resting and stimulated cells within experimental error. On the other hand, an increase in DMO counts in the cell pellet accompanies stimulation. If the explanation advanced by Villegas is correct, a change in wet/dry ratios would be predicted with no change in total DMO counts in the cell pellet. Furthermore, the active extrusion of H^+ ultimately requires the splitting of water into H^+ and OH^-, that would remain in the cell.

It is impossible to calculate the output of H^+ from the shift in intracellular pH. The magnitude of this change depends on the initial rate of H^+ secretion, the volume and buffering capacity of the cytoplasmic compartment, and the rate of alkaline release at the serosal side. This type of approach, however, may be useful if in a simple experiment relative rates of H^+ secretion are studied in response to different agents.

Thus, intracellular pH increases on stimulation according to what would be predicted if the cells secreted acid.

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CONCLUSIONS

The work described in this thesis shows that oxyntic cells can be isolated from the gastric epithelium and that these cells preserve most of the properties particular to that type of cell. Furthermore, the broad characterization of the isolated cell preparation gives substantial information about some of the cellular and subcellular processes in the stimulation, production, and regulation of H^+ secretion.

As a means of correlating the vast knowledge obtained from studies in the intact mucosa and new information obtained in this study on isolated oxyntic cells, I would like to propose a model describing the main events that lead to the secretion of hydrochloric acid by the oxyntic cell. Figure 35 schematically represents an oxyntic cell. Acetylcholine and gastrin liberate histamine from stores outside the oxyntic cell. Histamine activates a receptor on the surface of the cell. In addition, acetylcholine stimulates directly a receptor on the cell surface. It is possible that pronase unmasks receptors or activates them. Activation of these receptors leads to the stimulation of the adenylcyclase that catalyzes the formation of cAMP from ATP. Thus, cyclic-AMP is considered to be the intracellular mediator of the aforementioned hormones. The pathways for metabolism of cAMP are not shown in this figure for the sake of clarity, but it is assumed that cAMP is degraded in the reaction catalyzed by phosphodiesterase, and that theophylline inhibits this reaction.

Once cAMP is formed inside the cell, it may act in two ways. The first concerns the activation of the proton translocating mechanism. At present, its nature and the intermediate steps are not understood. The second consists of the activation of metabolic processes such as

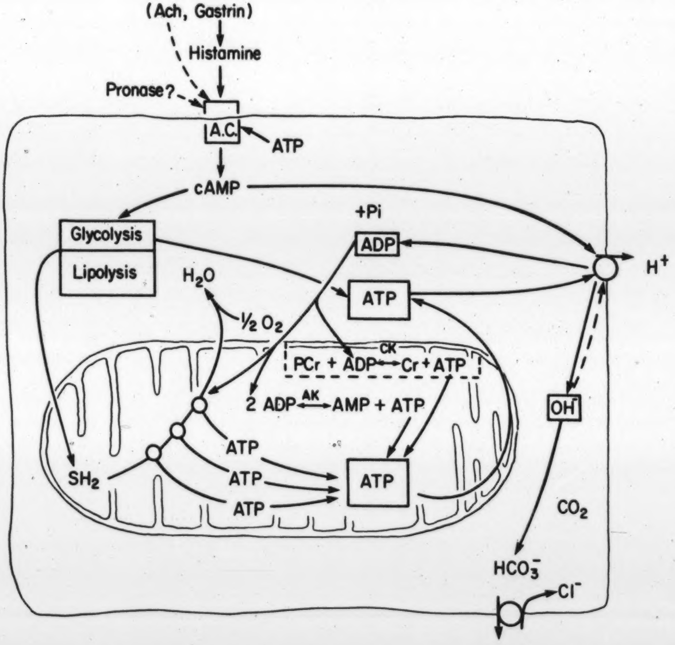

Figure 35: Schematic representation of an oxyntic cell, and some of the events taking place in the processes of stimulation, activation and regulation of the H^+ secretory process. See text for details.

glycolysis and lipolysis. Stimulation of glycolysis results from activation of phosphorilase, whereas stimulation of lipolysis occurs by means of the activation of lipase.

The activated H^+ secretory mechanism utilizes ATP from a cytoplasmic ATP pool as an energy source. This pool is generated from: 1) ATP synthesized in mitochondria; 2) glycolytic reactions; and 3) that fraction of the creatine kinase reaction localized in the cytoplasm.

The mitochondrial ATP pool is derived from: 1) oxidative phosphorylation; 2) that fraction of the creatine kinase reaction localized in mitochondria (40,130); and 3) the adenylate kinase reaction.

Conversely, ADP generated from the hydrolysis of ATP by energy consuming reactions such as the pump, can be rephosphorylated by the reactions mentioned above.

The rate of respiration depends on the availability to the respiratory chain of reducing equivalents provided mainly by lipolysis and, to a certain extent, by glycolysis, and by the accessibility of intramitochondrial ADP.

Mitochondrial and cytoplasmic pools of ATP and ADP are in equilibrium in resting conditions; possibly, however, these two are not in balance during the active secreting state. Alterations in the concentrations of these compounds found in the transition from the resting to the secreting state reflect the deviation from equilibrium of these reactions; however, they do not afford information concerning the rate of turnover.

The active secretion of protons leaves the cell with an excess of OH^- that reduces the activity of H^+ inside the cell. Modification of the activity of intracellular H^+ may serve as a feedback regulator of the rate of proton translocation. In this regard, it is interesting that acetazolamide inhibits acid secretion and raises intracellular pH (131). The excess base would be neutralized by CO_2 or other buffers that leave the cell at the serosal side in exchange for Cl^-. The way in which Cl^- is secreted at the luminal side is not discussed here.

Although this scheme is far from being proved, it raises further questions to be pursued.

Furthermore, it provides a model that describes certain aspects of the cellular mechanisms of HCl production and regulation explored in this thesis.

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